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INVESTIGATIONS INTO THE DISTRIBUTION OF BLOOD IN THE HEART AND AORTIC ARCHES OF *XENOPUS LAEVIS* (DAUD.)

BY THE LATE A. R. DE GRAAF*

Department of Zoology, University of Cape Town

(With Plates 3 and 4)

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I. INTRODUCTION

Except for typical fishes with their single circulation and post-embryonic birds and mammals with their complete double circulation, vertebrates possess an incomplete double circulation, having various degrees of anatomical division in the heart structure.

Anuran Amphibia are a case in point. They have, as is well known, a complete atrial septum, no septal division in the ventricle and only a partial division of the bulbus cordis by a spiral valve. Up to some twenty years ago it was generally accepted that, in spite of this imperfect separation, some physiological 'attempt' was made by the animal to aproach the perfect double circulation. The first reasoned account of the circulation of blood through the anuran heart was given by Brücke (1852). His ideas of the basic mechanism were modified and extended by Sabatier (1873), and have now become known as the 'classical theory'. This hypothesis may be briefly summarized (see also Foxon, 1955).

In spite of the absence of ventricular septum and only partial division of the bulbus cordis, there is a functional separation between oxygenated blood (from lungs to left atrium), and de-oxygenated blood (from body to right atrium). On atrial contraction these different types of blood would have little opportunity to mix in the ventricle on account of, first, the propulsion gained from the atrial contraction; secondly, the projection of the atrial septum down through the atrio-ventricular orifice; and thirdly, the simultaneous dilation of the ventricle which draws the blood into its trabecular meshwork. During ventricular contraction the first blood to leave is oxygen-poor blood, and this then passes into the cavum aorticum of the bulbus at pressure sufficient to distend the bulbus wall. The spiral valve does not now touch the opposite wall of the bulbus, and thus the blood has free access to the cavum pulmo-cutaneum. Since it is assumed that the peripheral resistance of the pulmo-cutaneous circuit is less than that of carotid or systemic circuits, an easier

* Adrian R. de Graaf presented a thesis embodying this work for the degree of M.Sc. at the University of Cape Town. He was awarded a Scholarship to proceed to Cambridge where he proposed to continue in the same line of research. His tragic death cut short a most promising career.

flow and a quicker reduction of pressure in the pulmo-cutaneous arches result. Thus, at the beginning of ventricular systole, oxygen-poor blood passes into the pulmo-cutaneous arches. At a later stage of ventricular systole the bulbus contracts, bringing the margin of the spiral valve into contact with the wall, thus completely separating the two cava of the bulbus. The cavum aorticum now receives the more oxygenated blood from the left side of the ventricle. This blood is channelled into the carotico-systemic arches. However, the blood does not enter the carotid arteries until at the last stage of ventricular contraction, for only then can the high resistance in these arteries be overcome. It is assumed in this connexion that the carotid labyrinths are the structures which help to maintain the high resistance.

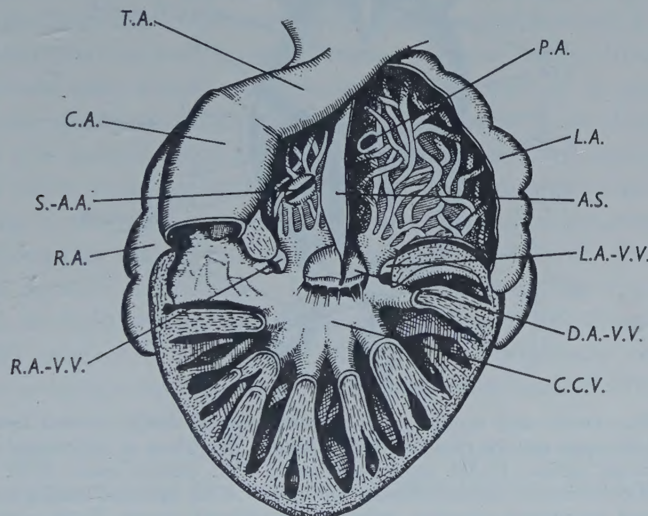
This 'classical theory' was derived originally from anatomical considerations and from direct visual observation on the beating heart. Apparently the only concrete evidence is the observation that in a pithed frog subjected to artificial respiration the blood entering the left atrium is lighter red in colour than that entering the right atrium. This distinction is maintained in the ventricle and the arterial arches, so that the pulmo-cutaneous arches receive the darker blood and the carotid and systemic arches the lighter. Gompertz (1884) found that during artificial respiration in *Rana* this division in coloration could be seen, whereas it promptly disappeared when respiration was suspended. Further, Ozorio de Almeida (1923) found a distinct line of division in the ventricle when he applied artificial respiration to *Leptodactylus ocellatus*. Noble (1925) injected indian ink into the pulmonary veins of a series of Anura and Urodela. He reports that in all species which frequently use their lungs and possess a functionally complete atrial septum and a spiral valve (*Scaphiopus holbrooki*, *Hyla crucifer*, *Acris gryllus* and *Rana clamitans*), none of the ink was passed into the pulmo-cutaneous arches. Acolat (1931*b*), working with *Rana* sp., replaced the blood by solutions of dyes and observed a clear separation in the ventricle. From further considerations (1931*a*, *b*, 1938*a*, *b*), however, he concluded that some mixture must take place. Simons & Michaelis (1953), using fluorescein injection, found selective distribution in two out of eight *Hyla caerulea*. Hazelhoff (1952) also advocated the 'classical theory'.

Other workers have criticized the 'classical theory'. As early as 1869 Fritsch claimed that the difference in coloration between the two sides of the heart and the different arches is solely due to difference in the transparency of the walls of the system. Gompertz (1884), although buttressing the 'classical theory', said that the spiral valve is at no time pressed against the opposite wall of the bulbus. Vandervael (1933) and Foxon (1947), using techniques similar to those of Noble on *Rana* and *Bufo*, maintained that the distribution is random, i.e. the blood from the two atria is thoroughly mixed by the time it leaves the bulbus cordis. Savolin (1949), injecting suspensions of starch or unicellular algae into the hepatic veins of *Bufo bufo*, also found an almost random distribution. Foxon (1948, 1951, 1953), applying a radiographic technique on *Rana temporaria* and *Bufo bufo*, confirmed the correctness of his earlier conclusions concerning random distribution. Finally, it must be mentioned that Simons & Michaelis (1953) found random distribution in several individuals of *Hyla caerulea*.

As can be seen, a relatively small number of species have been used for experimental work, and considering the disagreement about the distribution even in these, no conclusions may be drawn as to whether there are any specific differences or not. The few pressure recordings in the arches made with inaccurate technique by Gompertz (1884) and Acolat (1938*a*) do not help us. Neither do the considerations on cutaneous respiration of Vandervael (1933). The 'classical theory' is based on uncertain ground, and it is hoped that the following investigations on *Xenopus* will help to clarify the problem.

II. THE STRUCTURE OF THE HEART

Although full descriptions of the blood vascular system of *Xenopus* and its development are available (Millard, 1941, 1942, 1945, 1949), so far there has been no published account of the heart structure. Nevertheless, dissection and serial sections show a fairly close agreement with the descriptions of *Rana esculenta* and *R. fusca* (Gaupp, 1899).

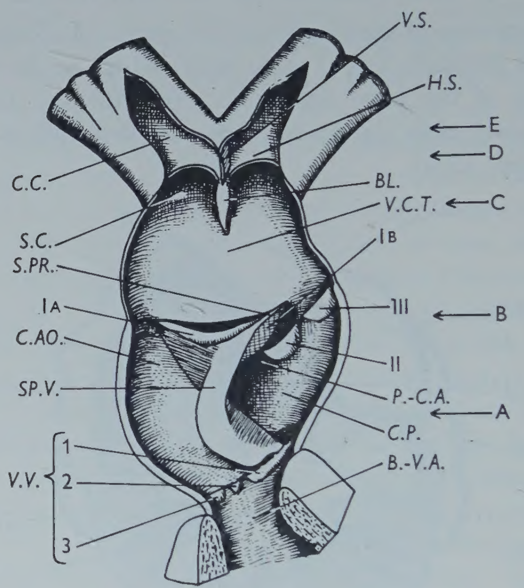


Text-fig. 1. Heart of *Xenopus leavis*, viewed from the ventral side. Ventral half of ventricle and ventral walls of atria removed. *A.S.* inter-atrial septum; *C.A.* conus arteriosus (bulbus cordis); *C.C.V.* central chamber of ventricle; *D.A.-V.V.* dorsal atrio-ventricular valve; *L.A.* left atrium; *L.A.-V.V.* left-atrio ventricular valve; *P.A.* pulmonary aperture; *R.A.* right atrium; *R.A.-V.V.* right atrio-ventricular valve; *S.-A.A.* sinu-atrial aperture; *T.A.* truncus arteriosus.

The sinus venosus is formed dorsally by the confluence of the anterior and posterior vena cavae and the large hepatic veins, and empties by way of a transversely oval aperture into the right atrium near to the mid-line (Text-fig. 1). The sinu-atrial aperture is guarded by anterior and posterior thickenings of the atrial wall, of which the anterior is the more prominent. These presumably act as valves, preventing reflux of blood into the sinus venosus. The pulmonary veins run forwards dorsal to the sinus and are closely bound to it. They unite to form a short common

trunk which empties into the left atrium close to the inter-atrial septum, but slightly anterior to the sinu-atrial aperture (Text-fig. 1). No valvular structures could be made out in the pulmonary aperture.

The two atria are completely separated by a thin, median, vertical septum and their interior surface, in the relaxed state, has a rugose appearance due to the projection of muscular bundles into the lumen. The position of the septum is not noticeable from the exterior, nor is there any external groove to indicate its line of attachment to the atrial wall. The margins of both atria, particularly where they overlap the ventricle ventrally, are partially subdivided into a series of pockets.



Text-fig. 2. Bulbus cordis and truncus arteriosus of *Xenopus laevis*, viewed from ventral side. Ventral wall slit open and the two sides pulled apart. *BL.* block of endothelial tissue; *B.-V.A.* bulbo-ventricular orifice; *C.AO.* cavum aorticum; *C.C.* carotid canal; *C.P.* cavum pulmo-cutaneum; *H.S.* horizontal septum (oblique septum); *P.-C.A.* aperture leading to dorsal chamber of truncus and to pulmo-cutaneous arches; *S.C.* systemic canal; *S.PR.* septum principale; *SP.V.* spiral valve; *V.C.T.* ventral chamber of truncus; *V.S.* vertical septum; *V.V.* 1, 2, 3 bulbo-ventricular valves; IA, IB, II, III valves at anterior end of bulbus. (A)–(E), levels at which sections shown in Text-fig. 3 were taken.

These are more prominent than diagrams and descriptions of the hearts of *R. esculenta* and *R. fusca* would indicate. Unlike *Rana*, *Xenopus* shows no distinct difference in anatomical size between the two atria.

The large atrio-ventricular orifice is guarded by two large, thick valves dorsally and ventrally. These valves are attached to the wall of the orifice anteriorly, but posteriorly have a number of chordae tendinae passing down and inserting into the ventricular musculature. Hardly to be described as semi-lunar, they are quite bulky in appearance, and have the same peculiar histological structure as the spiral valve of the bulbus cordis, which is presumably composed of hardened and modified

endocardial tissue. On either side of the atrio-ventricular orifice is a weakly developed, semi-lunar valve (Text-fig. 1).

Posteriorly, the inter-atrial septum is fused with the middle of each of the large dorsal and ventral valves and projects for a short distance into the lumen of the ventricle.

The ventricle has a clear central chamber of relatively small size, into which the atrio-ventricular orifice leads, but the rest of its interior is broken up by the crossing of a large number of muscular trabeculae which, in section, give the appearance of a thick spongy wall. The outer wall of the ventricle, however, is relatively thin. From the right dorsal part of the central chamber an aperture leads into the bulbus cordis (Text-fig. 1). The latter is provided at its base with a series of three semi-lunar valves, of which the largest is ventral and the other two are smaller and dorso-lateral in position. Attached ventrally to the bulbus wall within the free margin of the ventral valve is the base of the large spiral 'valve'. This valve then follows a spiral course round the wall of the bulbus in a clockwise fashion when seen from the ventricle, until at the anterior end it is attached to the right side, having turned through about 270°. Although at the posterior end it is only a comparatively small projection from the wall, it rapidly increases in size and, in dissections of fresh material, it would seem almost to fill the lumen of the bulbus. In serial sections its free margin extends about two-thirds across the bulbus for most of its length (Text-fig. 3A), but it is quite obvious that some shrinkage occurred during preparation.

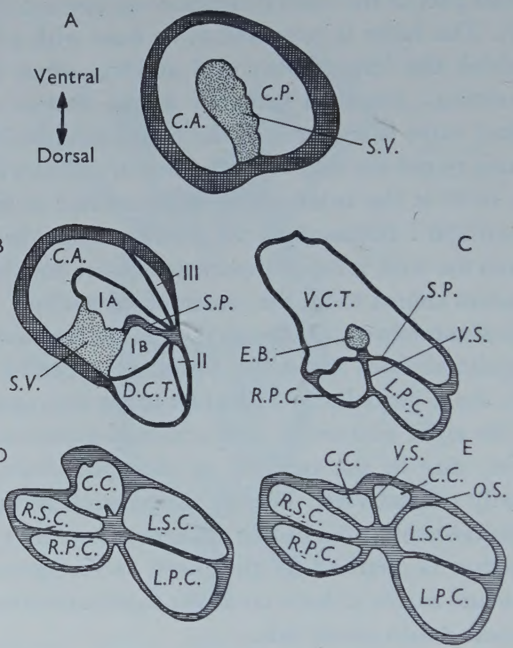
The spiral valve, then, partially divides the bulbus into two channels, a cavum aorticum lying to the right posteriorly and a cavum pulmo-cutaneum to the left. Anteriorly, however, due to the twisting of the spiral valve, these come to lie ventrally and dorsally respectively. Due to the position of the spiral valve at the posterior end, the bulbo-ventricular orifice appears to lead into the cavum aorticum only, and this junction is guarded by the three valves already mentioned. The cavum pulmo-cutaneum seems to have no direct communication with the ventricle, although there is some doubt about this.

Anteriorly, the bulbus is followed by a thinner-walled tube, the truncus arteriosus, which is completely divided by a horizontal septum (septum principale) into dorsal and ventral chambers, corresponding to cavum pulmo-cutaneum and cavum aorticum of the bulbus. This septum is continuous behind with the spiral valve (Text-fig. 3B, C). At the anterior end of the bulbus is a series of semi-lunar valves, guarding its exit to the truncus. Two of these (valves IA and IB of Gaupp, 1899) are attached to the spiral valve and have their free margins projecting into the cavum aorticum and cavum pulmo-cutaneum respectively, while the others (valves II and III) are attached to the bulbus wall and lie opposite the first two (Text-figs. 2, 3B).

Slightly anterior to the bulbus, the dorsal chamber of the truncus is split into right and left pulmo-cutaneous canals by a vertical septum (Text-fig. 3C). Still farther forward oblique and vertical septa almost simultaneously divide the ventral chamber of the truncus into four canals, which continue to form the paired systemic and carotid arches (Text-figs. 2, 3D, E). This is somewhat different from the arrangement in *Rana*, where both carotid canals arise from the right side of a main vertical

septum, but it resembles the condition in urodeles and also in *Microhyla* and *Ramanella*, described by Rao & Ramanna (1925).

Projecting posteriorly from the vertical septum into the ventral chamber of the truncus, and attached to the ventral surface of the septum principale, is a block of tissue similar in composition to the spiral valve. This partially divides the chamber at its anterior end into two divisions, leading to the systemico-carotid canals on each side (Text-figs. 2, 3 C). No valvulae paradoxae could be found in the systemic canals of the truncus or in the systemic arches themselves.



Text-fig. 3. Selected sections through the bulbus cordis and truncus arteriosus of *Xenopus laevis* (semi-diagrammatic). (A) section through middle of bulbus; (B) through junction of bulbus and truncus; (C) through truncus before origin of carotid and systemic canals; (D) and (E) through truncus in successively more anterior planes. Level of sections indicated in Text-fig. 2. *C.A.* cavum aorticum; *C.C.* carotid canals; *C.P.* cavum pulmo-cutaneum; *D.C.T.* dorsal chamber of truncus; *E.B.* endothelial block in ventral chamber of truncus; *L.P.C.* left pulmo-cutaneous canal; *L.S.C.* left systemic canal; *O.S.* oblique septum; *R.P.C.* right pulmo-cutaneous canal; *R.S.C.* right systemic canal; *S.P.* septum principale; *S.V.* spiral valve; *V.C.T.* ventral chamber of truncus; *V.S.* vertical septum. IA, IB, II, III, valves at anterior end of bulbus.

A coronary artery arises from the base of one of the carotid canals of the truncus and runs backwards across the surface of the bulbus, but reaches no farther. Venous drainage from the wall of the bulbus passes into a coronary vein, which runs down laterally between the ventricle and right atrium to join the sinus venosus. There is no coronary supply to the rest of the heart.

III. PHYSIOLOGICAL INVESTIGATIONS

(1) *Distribution of blood*

A. *Methods*

The basic question as to whether or not there is a selective distribution of blood would seem to be the one most sorely in need of clarification. Most attention has therefore been given to this aspect of the problem. Several methods are available for this study.

Measuring the oxygen content of the blood arriving at, and departing from, the heart by various channels, has not yet been employed. The small quantities of blood which can be removed without risking disturbance in the pressure conditions of the system make an approach along this line very difficult.

Injection and recovery of some identifiable substance (Savolin, 1949) does not permit the tracing of substances through the ventricle and bulbus, although it is possible, in principle, to obtain quantitative results.

Injection of radioactive substances and their tracing is an obvious possibility. Simons (personal communication), however, has attempted the method without success. The main difficulty, apparently, is to obtain a great enough concentration in the arterial arches to give significant measurements.

The radiographic method used by Foxon (1951) is limited by a slow frequency of photographic exposure, due to the necessity of moving large masses of sensitive material, and of using lead shutters. In addition to this there are difficulties which prevented Foxon from obtaining more than about 20% 'satisfactory' results.

Cinematographic tracing of injected fluorescein was used by Simons & Michaelis (1953). This technique does not suffer from the disadvantages encountered by Foxon. Both methods, however, allow one to trace the injected material through the heart itself.

All in all, the fluorescein technique seems the most promising one, and this was the reason why we concentrated on this method, adapted from Simons & Michaelis (1953). Certain precautions are necessary in order to avoid the possible introduction of considerable errors.

First of all the animal must not lose much blood during the experiment, since this would upset normal pressure conditions. This does not seem to have bothered other workers. Vandervael (1933), in fact, considers the blood loss and slowing of the heart resulting from pithing as advantageous, in that the action of the heart could more readily be observed!

Secondly, the amount of material injected should not cause an appreciable rise in pressure in the injected vessel; Foxon (1951) states that for a thorotrast solution to be discernible in the systemic arch it had to be in a concentration of at least 25%. Considering the dilution of the medium in its passage through the heart, it would be necessary to inject undiluted thorotrast into a pulmonary vein in quantities at least equal to the volume of blood normally flowing through this blood vessel. Simons & Michaelis (1953) found it necessary to inject volumes of about 1 ml. fluorescein. Such quantities, apart from changing the viscosity of the blood, increase the blood

volume and thus change pressure conditions quite considerably, with unpredictable results.

Thirdly, the injected substance should mix easily with the blood and not form a separate viscous mass. Foxon (1951) points out how this may affect the blood circulation. One of the reasons for the failure of some of his experiments, he states, was that the thorotrast interfered with the action of the atrio-ventricular valves, and on contraction of the ventricle it was regurgitated into the atrium.

Finally, the injected substance should exert no chemical effect on the heart. Fluorescein, as is well known, is physiologically neutral and has been used for a range of physiological experiments, even on man.

In addition, it may be mentioned that the animal should be subjected to a minimum disturbance from normal conditions, or should be subjected only to disturbances which may be determined and taken into consideration. For most injection techniques it is necessary to expose the heart. Insufficient attention has been paid to the abnormalities which may result from this procedure. Unfortunately, it is difficult to determine the possible deviations in question. Some attention has been given, in the present investigations, to the effect of opening the body cavity. This operation causes the removal of pressure exerted on the internal organs by the abdominal musculature and thus might lead to changes in the blood pressure of the pulmonary circuit. However, observations on animals in a pressure chamber, and with distended lungs, showed that no significant changes in blood pressures or pulse curves occurred if the toads were not subjected to completely unphysiological pressure conditions.

The following techniques were adopted for our purposes:

Preparation of toads. In most cases a preliminary subcutaneous injection of about 0·6 ml. of 20% ethylurethane was given as an anaesthetic. As is well known, this has the effect of dilating the blood vessels and would thus cause a general lowering of the pressure in the system. In other cases, therefore, unanaesthetized toads were used as controls. The experiments showed that urethane had no apparent effect on the type of result obtained.

The toad was fixed to a cork mat, the abdomen opened, and a median incision made through the pectoral girdle. The two sides of the pectoral girdle were then loosely pinned back to expose the heart and arterial arches. For injections into the pulmonary vein, or for observing the circulation through a lung, the latter was cleared of the mesenteries and the lung gently moved to one side. When carefully done, the blood loss resulting from these operations was insignificant. All exposed parts were kept moist during the course of the experiment with drops of Ringer solution. Female *Xenopus* were found to be unsatisfactory subjects because the pulmo-cutaneous arch was usually hidden, and the carotid arch was also frequently in an unsuitable position for photography. Thus male toads of 40–50 g., having a much more favourable arrangement of the arterial arches, were used in almost all cases. The injections and photographic recording were carried out with the toads lying horizontally. Before the injections were started care was taken to verify that there was no interference with the normal free flow of blood in the vessels leading to

and from the heart, and that the heart was not beating in an obviously abnormal fashion.

Injection method. An Agla micrometer syringe was used as the injection unit. By fixing a length of thin lead tubing on the end of the barrel of the syringe and a very fine cannula (record no. 21) at the other end of the lead tube, the system was sufficiently flexible for adjustment of the cannula position without elastic recoil. This appeared to eliminate difficulties encountered by Foxon (1951), who frequently introduced air into the blood vessel at the same time as his contrast medium. The cannulae used were found to be small enough to allow insertion even into the pulmonary veins of the smaller specimens, without blood loss and without materially obstructing the flow of blood. The syringe itself was fixed in a clamp.

Various concentrations of fluorescein–Ringer solutions were tried, the most satisfactory being a 1 % solution. At concentrations higher than this, the fluorescein was found to diffuse into the blood before the actual injection was started, and this led to a preliminary fluorescence of the heart and aortic arches. The amounts injected varied between 0·15 and 0·004 ml., the average figure being 0·046 ml. It cannot be supposed that such small volumes could have any significantly adverse effect on the blood circulation, either through raising the pressure or by decreasing the viscosity of the blood. The solution was injected slowly and continuously during each experiment.

Illuminant. Ultra-violet illumination was provided by three or four 125 W. Philips mercury-discharge lamps, type 57202 E/70, mounted in polished aluminium reflectors. At a distance of about 1·5 ft. these reflectors focused the light into an intense bluish patch of about 2 in. diameter. Any unevenness of illumination was counterbalanced by the superimposition of the other lamp's brightest areas. Since the fluorescein solution was strongly fluorescent to the eye, both in the brightest patches of focused light and immediately outside them, it seemed unlikely that the passage of any fluorescein would not be recorded by the camera, due to inadequate ultra-violet irradiation in some areas.

Ultra-violet penetration of the aortic arches. To test for any possible differences of penetration of the ultra-violet light through the walls of the different arterial arches, the following check was made. Small sections of the arches were removed from a toad, washed out with Ringer solution and slipped over the end of a glass tube of bore approximately equal to that of the arch in its normal state. Various concentrations of fluorescein–Ringer solution were then drawn up into the tube and exposed to the camera under ultra-violet light. The fluorescein solution was clearly visible on the negative down to a concentration of 1:10,000. It is true that the sections did not always transmit the fluorescence with equal intensity. This can probably be explained by the difficulty of ensuring that each of the sections was stretched to the same extent. Histological examination of the walls of the arches showed no significant differences in structure or thickness, and it was considered that, since such low concentrations of fluorescein gave a distinct record, the relative penetrability of the blood vessel walls need not be considered in the evaluation of qualitative results.

Filming. For photographic recording a Paillard Bolex 16 mm. camera with a parallax adjusting mechanism was employed. Using a 75 mm. lens with a short extension of 7 mm., and a 2-dioptre accessory lens, a field of about $1 \times 1\frac{1}{2}$ in. could be photographed at an aperture of *f* 3·5 with sufficient depth of focus for clarity. In order to eliminate reflected ultra-violet light a Kodak Wratten K-2 gelatine filter was used, and in addition a Voigtländer G-2 yellow filter to eliminate the visible bluish light emitted by the lamps. Kodak Super XX panchromatic film was found to be eminently suited to the purpose, since it was possible to photograph at a speed of up to 24 frames per second and more. The films were slightly overdeveloped with Ilford ID-11 M.Q. Borax fine-grain developer. In this way it was possible to obtain negatives of quite satisfactory contrast. The field covered included the heart, main arterial arches and some surrounding tissue. A certain amount of the fine detail was lost, especially movements of the bulbus. Attempts were made to obtain a more detailed idea of the sequence of events, but it was found that the depth of focus was so much restricted by narrowing the field that the results could not be interpreted. As will appear, the main features exposed by the technique were the filling of the atria and ventricle, and the distribution of the fluorescein to the arterial arches.

B. *Results of injection experiments*

(*a*) *Injections via the right atrium.* Injections were made into the left anterior vena cava or into one of the hepatic veins. A typical recording is reproduced in Pl. 3, being one out of fourteen similar recordings made.

The injection site and the proximal portion of the vein was always marked by a dark mass, due to the accumulation of fluorescein solution as a result of the slow movement of blood in these large vessels (Pl. 3, *1*). The subsequent course of events was somewhat variable, although not different in principle. After one or two heart beats, the fluorescein might become visible first in either the right atrium or the ventricle, or both simultaneously. In Pl. 3, *1–3*, the right atrium does not fill completely with fluorescein at its first diastole, although atrium and ventricle start to fluoresce simultaneously. In all cases, the brilliance of the fluorescence took some time to build up in the atrium. The marginal pockets in particular never obtained a full coloration until a few heart beats had occurred. Passage of the fluorescein into the sinus venosus or between the atrio-ventricular valves was obviously not recorded (due to the thickness of the heart wall?).

In ten cases the appearance of fluorescein in the ventricle was only on the right side, with a more or less sharp line of division between this and the clear left side (Pl. 3, *1–12*). This discriminating coloration is interpretable as being due to the penetration of the fluorescein-laden blood in the intertrabecular spaces near the surface of the ventricle. In four cases the line of demarcation between the two sides was not sharp, only a small portion of the ventricle on its extreme right or at its apex becoming coloured. This may have been due to insufficient penetration of the dye into the trabecular meshwork.

During contraction of the ventricle the fluorescence remained, since some fluoresceinated blood had been retained in the muscular meshwork (Pls. 3 and 4).

Foxon (1951) has proposed that this remnant, which he found in *Rana* and *Bufo*, might be blood concerned with the supply of oxygen to the ventricular musculature.

The visible division of the ventricle was obscured some three or four beats after its first appearance, when the left side also became coloured. Sometimes this occurred quite suddenly in the space of one ventricular diastole (Pl. 3, *12–14*), or sometimes more gradually, and is associated with the recirculation of fluorescein through the pulmonary circuit into the left atrium. This is proved by the fact that the division never breaks down before the pulmonary circuit, as shown by lung and pulmonary vein, has obtained a bright fluorescence. In Pl. 3, for example, the lung starts to fluoresce at frames *6–8*, the pulmonary vein at *10*, the left atrium at *11–12*.

On the first or second heart beat, but not later, the fluorescein was quite distinctly detectable in the pulmo-cutaneous arches. No sign of it, however, appeared either in the ventral chamber of the truncus, carotid or systemic arches at that time. This can be clearly seen in Pl. 3, at the first ventricular systole (frames *3–6*), fluorescein appears very darkly in the pulmo-cutaneous arches only. The *distinct* appearance of fluorescein in the ventral chamber of the truncus was later, usually delayed until the third, fourth, or later heart beat (Pl. 3, *12*), i.e. when there was already a coloration of the left side of the ventricle, or there was at least the probability of recirculation of fluorescein through the pulmonary circuit.

In most cases, the pulmo-cutaneous arches remained distinctly more fluorescent throughout the length of the record (e.g. Pl. 3). In two cases the systemic or carotid arches attained a similar density at the fifth or sixth heart beat. In Pl. 3 no appreciable quantity of fluorescein is passed into the systemic and carotid arches until the third beat, when recirculation of fluorescein through the pulmonary circuit has occurred. Only a little fluorescein is visible in the systemic (particularly the right one) and carotid arches during the second beat. It is noticeable that the fluorescein in the right systemic arch only reaches about half-way up the visible part of that arch during the second ventricular systole (*9–10*), but progresses the rest of the way in the third ventricular systole (*12*). This should be compared with the complete filling of the visible part of the pulmo-cutaneous arch at the first ventricular systole (*3–6*). In all cases the maximum density of fluorescence in the pulmo-cutaneous arches was reached by the second or third heart beat. It must be mentioned, however, that the arches did not always reach the same brightness on both sides. It is likely that some of the arches were shaded from ultra-violet light in these cases, due to the position of the heart, for it was not a constant occurrence. The fluorescein sometimes appeared first in the form of streaks in the arterial arches (e.g. Pl. 3).

The speed of flow of blood through the pulmo-cutaneous arches and the pulmonary artery was obviously high, for the fluorescein appeared in the lungs at the first or second beat. In contrast to this there was never any recordable fluorescence in the tissues supplied by the carotid or systemic arches before the seventh or eighth heart beat.

Attention must be drawn to an aspect of fluorescence of the bulbus in relation to statements by former workers. In these experiments the main part of the bulbus

visible from the ventral surface was that part which corresponds to the cavum aorticum. At most, only a small section of the cavum pulmo-cutaneum would be visible at the base and on the left side of the bulbus, from this angle. This would be separated from the cavum aorticum by the free margin of the spiral valve, which follows a diagonal line from the bottom centre to the top left part of the visible region of the bulbus. The rest of the cavum pulmo-cutaneum would be hidden beneath the spiral valve. With these structural arrangements in mind, it must be noted that in most cases either no coloration of the bulbus was visible until the third beat, or the anterior part was clear while a dark basal part was discernible with a diagonal border (which may well be interpreted as the free margin of the spiral valve). This state of affairs is consistent with the supposition that the fluorescein-laden blood from the right side of the ventricle was either passing directly into the cavum pulmo-cutaneum, or was traversing only the lower part of the cavum aorticum before passing over the spiral valve into the cavum pulmo-cutaneum. In four cases, however, a 'pulse' of fluorescein was visible which in one case clearly passed through the bulbus from the right to the left side (Pl. 3, *3* and *8*, arrows). During this time no great fluorescence appeared in the ventral chamber of the truncus or in the four anterior arches. It would therefore appear that blood may be admitted to the cavum pulmo-cutaneum by first travelling higher up the cavum aorticum with subsequent leakage over a more anterior part of the spiral valve. These observations are placed on record as an indication that the statements by Ozorio de Almeida (1923), Vandervael (1933) and Foxon (1947), that the blood passes up as two *separate* streams in the cava, do not always apply in *Xenopus*, since blood can pass over the spiral valve from the cavum aorticum to the cavum pulmo-cutaneum.

(*b*) *Injections via the left atrium.* Injections were made into the left or right pulmonary vein. A typical recording is reproduced in Pl. 4, one out of eighteen cases. Certain significant differences emerged from the results of injections into the pulmonary veins. Where no differences are remarked upon in what follows, the picture was the same as with injections via the right atrium.

In the records, the pulmonary vein injection site was not always clearly visible either because this was not included in the area filmed or because it was hidden by the heart. When, however, the site of the injection could be seen, it showed that the injected solution was rapidly washed away by a wave of non-fluoresceinated blood at each atrial diastole, in contradistinction to injection into a vena cava or hepatic vein. This indicates that the flow through the pulmonary veins was considerably faster than that through the anterior vena cava or a hepatic vein.

There was again a gradual filling of the marginal pockets of the atria over a number of heart beats (Pl. 4). The initial filling of the ventricle was somewhat variable. In the majority of experiments (thirteen cases) either the whole ventricle appeared coloured at the first beat (e.g. Pl. 4, *1–3*), or spots appeared on both sides at the first beat, denser on the left side. In four other cases only the left side of the ventricle was coloured at the first beat. Two of these cases showed a line of demarcation between right and left sides which lay well to the right of the median line. When a division occurred this tended to disappear rapidly. Unlike the injections via the

right side, this was not to be correlated with a recirculation of fluorescein which only occurred much later.

The pulmo-cutaneous arches showed coloration at the first beat in fifteen cases (Pl. 4, *4–13*), and at the second beat in three cases. In twelve cases coloration of the bases of the carotid and systemic arches was simultaneous with that of the pulmo-cutaneous arches (Pl. 4, *5–6*), in five cases a little after, and in one case slightly earlier than the pulmo-cutaneous arch coloration. In fifteen experiments the pulmo-cutaneous arches were more fluorescent than the others for at least the first four beats (Pl. 4, *4–18*). In four records they even remained darker throughout the length of the film strip (six to ten heart beats). This indicates that the pulmo-cutaneous arches were receiving a considerable proportion of the fluorescein injected into the pulmonary vein. Fluorescence of the lungs (Pl. 4, *8*, *9*) and pulmonary veins occurred at about the same time as with injections in the hepatic vein, but coloration of other tissues such as the distributional area of the external carotid artery tended to occur sooner than by injections via the right atrium. The cavum aorticum of the bulbus was usually fluorescent from the first beat, or at least from the time of the first appearance of dye in the ventral chamber of the truncus.

C. *Discussion of results of injection experiments*

The results obtained lead to several conclusions discussed below:

(1) Upon injection via the right atrium a division in the ventricle appears, so that only the right side is fluorescent until the division is obscured by recirculation of the dye. However, a note of caution is advisable. The line of demarcation as shown in the records represents only a surface picture of the ventricle. In the fluorescein technique a picture of the *whole* ventricle is not obtained, since both the ultra-violet light and the resulting fluorescence can only penetrate a short distance through the ventricular trabeculae, and the blood in between them. The line of demarcation between fluoresceinated and non-fluoresceinated blood, through the depth of the ventricle, might either be in a vertical plane or might incline considerably in a diagonal fashion. Yet it is not to be expected that the line of division would differ much from the vertical plane in which the inter-atrial septum projects into the central chamber of the ventricle. The sharp median line of demarcation between light and dark sides of the ventricle appearing in many of the records is therefore interpreted as being due to absorption of fluoresceinated blood between the trabeculae on the right side only. It is clear, therefore, that a form of division between the blood from left and right atria occurs in the ventricle, in so far as right atrial blood is absorbed only into the trabecular meshwork on the right side of the ventricle.

(2) Upon injection via the left atrium the fluorescein either tends to spread all over the ventricle at once, or if, exceptionally, a division in the ventricle occurs, the line of demarcation may lie well to the right side of the median line, and anyway disappears before the commencing fluorescence of the right side can be attributed to recirculation. Comparing this with the results obtained with injection of the right side, the following statement can be made: Whereas the output of the right atrium

is not, or only in negligible quantities, transferred to the left side of the ventricle, the output of the left atrium shifts, to a considerable extent, to the right half of the ventricle.

(3) By comparing injections via left and right atrium it can be seen that the left atrial output occupies more ventricular space than the right output (see, for example, Pls. 3 and 4). The blood from the left atrium either fills the left half and, moreover, some of it mingles with the right-side blood, or, if there is an initial separation, the blood of the left side may extend considerably into the right side. In contradistinction to this the output of the right atrium does not occupy more than the right half of the ventricle. We, therefore, draw the following conclusion: the left atrium has a larger output than the right atrium. No anatomical data conflict with this statement.

(4) After injection via the right atrium the pulmo-cutaneous arches fluoresce distinctly within a short time, but no significant coloration of the systemic and carotid arches occurs before recirculation of the die commences. It is true that in some experiments a faint tingeing of the systemic and/or carotid arches did appear before it could be said with certainty that recirculation had occurred. However, one cannot expect that a heart without complete anatomical division can possess a complete selective distribution. It is clear, anyway, that almost all the blood expelled from the right atrium is sent to the pulmo-cutaneous arches.

(5) After injections via the left atrium fluorescein tends to appear simultaneously in the bases of all the arches at the first beat, i.e. the appearance of dye in the carotid and systemic arches now tends to coincide with its appearance in the pulmo-cutaneous arches. In addition, it must be stressed that the pulmo-cutaneous arches were more fluorescent than the others for at least the first four beats. In contradistinction to the flow of the output of the right atrium, the blood from the left atrium is distributed to all the arterial arches, and the pulmo-cutaneous arches receive a considerable proportion of this blood.

(6) By comparison of the film recordings (see, for example, Pls. 3 and 4) one obtains the strong impression that, at the very least, three-quarters of the right atrial blood plus one-third of the left atrial blood is received by the pulmo-cutaneous arches. Since the left atrium has a larger output, these approximate figures do not necessarily indicate that the pulmo-cutaneous arches receive more than half of the blood from the heart. However, other considerations lead to that conclusion: the left atrium has a larger output, output must be equal to input, thus the pulmonary veins must supply the left atrium with more blood than delivered by the sinus venosus to the right atrium. Consequently the capillary bed of the lungs, and hence the pulmonary arteries, must carry more blood than the body circuit. Since the pulmonary arteries transport the blood delivered by the pulmo-cutaneous arches minus the blood which is diverted to the cutaneous arteries, the pulmo-cutaneous arches must absorb, very definitely, more blood than the other arches together. The drainage by the cutaneous arteries, and the supply through the cutaneous veins to the right atrium (via anterior venae cavae) is not very considerable. The cutaneous arteries are very small in *Xenopus*, having as measured, in fresh preparations, about

one-ninth of the cross-sectional area of the pulmonary arteries. Nevertheless, some drainage of the pulmonary system will occur. All in all, it seems safe to conclude that more blood is flowing through the pulmo-cutaneous arches at each beat than is being sent through the carotid and systemic arches together.

Additional evidence is produced by observations of the velocity on blood flow. In all cases the fluorescein passed up the whole visible part of the pulmo-cutaneous arch during the same beat at which it appeared at the base of the arch. It was visible in the lungs at about the second beat, and in the pulmonary vein at about the third beat. This indicates a very rapid rate of flow through the pulmonary circuit. By comparison, the impressions gained of the speed of flow in the systemic and carotid arches are as follows.

In no case was fluorescein visible at the ends of the exposed portions of the carotid or systemic arches before the second beat after its first appearance at the base of those arches (the length of the exposed portions of all the arches were roughly equal). Usually it took some three or four beats for the dye to reach this distance. In some instances the dye could be seen to progress a short distance up the arch with each beat, although no clear wave front was visible. The appearance of fluorescein in the peripheral distributional area of the carotid or systemic arches occurred later than in the lungs. In no case was any recirculation of fluorescein visible in the right atrium during the course of pulmonary vein injection experiments, even when these lasted for seventeen or eighteen heart beats. In fact, from direct visual observation, return of fluorescein through the anterior venae cavae was not detectable before about the twenty-fifth beat.

The much slower rate of flow in the systemic and carotid vessels could also be seen by direct observation of the blood corpuscles through the arterial walls, under a dissecting microscope, with strong side illumination. In the carotid and systemic arches these movements took place in a jerky fashion, consisting of three phases. First, a sudden rapid movement over a short distance which corresponded with ventricular systole, subsequently a brief complete halt or nearly so, followed by an increasing and moderate speed of flow, which appeared to correspond with contraction of the bulbus. It is significant that by using the same method the movement of corpuscles in the pulmo-cutaneous arches could not be observed, until some obstruction was applied, i.e. the blood was moving too fast all the time for the corpuscles to be visible.

The speed of flow in the pulmo-cutaneous vessels must be at least twice as high as in the other arches, which strengthens the conclusion that they are dealing with more blood per beat than the rest of the system.

(2) *Pressure measurements in the blood vessels*

A. *Methods*

Only two references have been found to previous experimental work in which simultaneous pressure measurements have been made in the pulmo-cutaneous and systemic or carotid arches of any anuran (Gompertz, 1884; Acolat, 1938*a*). This is rather surprising considering the importance of pressure differences in the

mechanism proposed by the 'classical theory'. Both workers used mercury manometers which, however small, have too low a frequency response to give an accurate idea of the shapes of the pulse curves or their amplitude. Moreover, their results do not agree with one another. Repetition of these experiments with *Xenopus*, using more adequate methods, was therefore an obvious necessity.

What was required for the present experimental work was a form of apparatus which would create as little disturbance in the animal as possible, record pressures accurately, and respond rapidly and faithfully to sudden pressure changes. The mercury manometer does not fulfil these requirements, and thus could not be considered for the present investigations. The optical manometer, operating by transmitting pressure changes to an elastic membrane on which a small mirror is fixed, seemed much more promising. Wiggers (1928) and Hamilton (1946) have published accounts of the mathematical principles of such systems. The Wiggers universal manometer has the disadvantage of requiring the use of a wide cannula which is obviously out of the question with animals as small as frogs. Hamilton, Brewer & Brotman (1934) and Gregg, Eckstein & Fineberg (1937) have described instruments which overcome this difficulty. In these the adverse effect of a narrow hypodermic cannula is counterbalanced by the use of a more rigid elastic membrane of small area.

In pressure transducer units the distortion of a membrane is measured by electrical means through a Wheatstone bridge and amplification system. One form of pressure transducer produced by the Statham Laboratories, California, was tried, but was found to be unsatisfactory, since it was not really designed for use with very small cannulae, or such low-pressure ranges. An optical manometer system was therefore used in the present investigations.

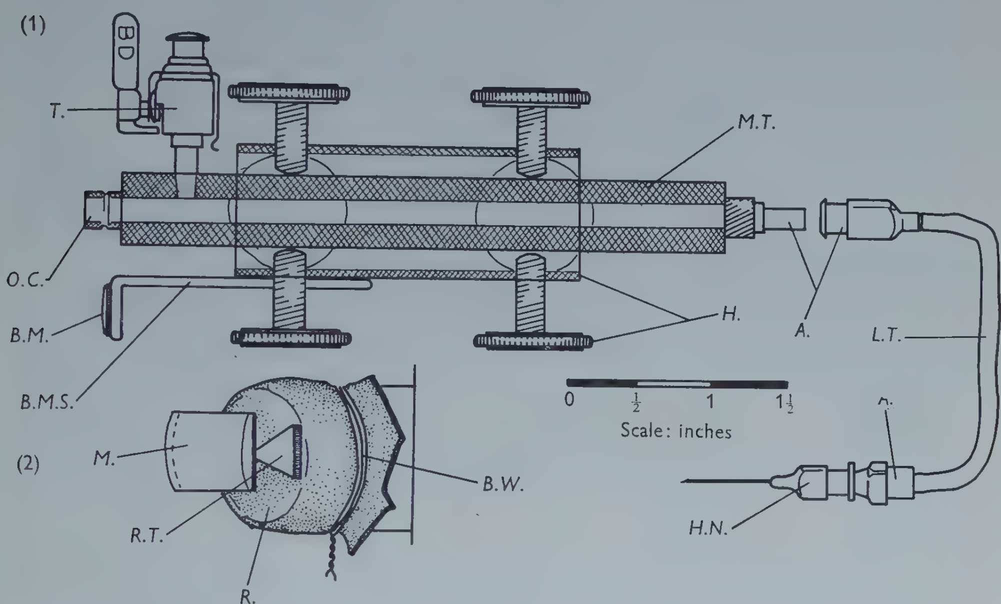
The design adopted was essentially the same as that described by Hamilton *et al.* (1934), but with a few modifications (Text-fig. 4). Two manometers were constructed, as identical as possible. A few brief comments follow on features differing from the Hamilton manometer.

The most satisfactory membrane was found to be rubber sheeting 0·7 mm. thick. As Gregg *et al.* (1937) point out this has the advantage that the excursion of the optical lever is linearly related to the pressure applied. The rubber sheeting was tied with wire over the end of the manometer tube, making sure that it was stretched equally in all directions, and to the same extent in the two manometers. On this was mounted a small triangle of the same rubber, glued with two points on the rim of the manometer tube and one point in the centre. The mirror, made from an 0·75 dioptre plano-convex wafer lens silvered on the flat side and cut down to a 5 mm. square, was mounted on the rubber triangle. This allowed the mirror to move on the segment principle (see Wiggers, 1928) in the horizontal axis only. As a precaution against misinterpretation of accidental jolting, base-line mirrors were attached to the outer housing.

The cannulae used were $\frac{1}{2}$ in., no. 20 hypodermic needles which were of a diameter small enough to be inserted into the arterial arches, without disturbing the normal flow of blood significantly. These were somewhat smaller than those used

by Hamilton or Gregg and their associates, and had the effect of overdamping the system, as will be seen.

The manometers were filled with a boiled 5 % sodium citrate solution, great care being taken to avoid air bubbles. To make quite sure that all bubbles were eliminated before each series of experiments, the whole manometer and lead connecting tube was treated in a vacuum chamber until the citrate solution boiled for some time.



Text-fig. 4. Optical manometer. (1) Whole manometer; (2) detail of optical capsule. *A.* adaptors soldered to manometer tube and lead tubing; *B.M.* base-line mirror; *B.M.S.* base-line mirror support; *B.W.* brass wire; *H.* adjustable housing; *H.N.* hypodermic needle; *L.T.* lead tubing; *M.T.* manometer tube; *M.* mirror; *O.C.* position of optical capsule; *R.* rubber membrane; *R.T.* rubber triangle mounted on membrane; *T.* tap.

Illumination was provided by a microscope projection lamp with a Philips 8 V. 6 amp. globe which could be overloaded to 10 V. An adjustable vertical slit in front of the lens of the lamp allowed a series of beams to be cast out from the vertical filaments of the lamp, and these could be trained on the mirrors of both manometers at the same time.

Recordings were taken with a photo-kymograph, constructed to a design by Prof. R. Goetz of the Surgical Research department of our University.

In order to check the inertia of the system tests were carried out at intervals, using the method described by Hamilton *et al.* (1934). Although the manometers followed a sudden change in pressure very rapidly, they were aperiodic, due to the damping effect of the fine cannulae. Various ways of overcoming this were tried. Increasing the size of the cannulae to no. 18 had no appreciable effect, and in any case these were too large to use without disturbing the blood flow. Increasing the

thickness of the rubber membrane cut down the sensitivity to such a degree that the optical lever had to be extended to an unwieldy length and involved difficulties in obtaining a sharp record of sufficient intensity. Stretching the membrane more tightly had no appreciable effect. However, it was felt that, considering the quick response which the manometers showed to the sudden rise in pressure in these tests, giving an almost square wave form to the recording (ascending slope lasting 0·2 sec., descending slope 0·05 sec. when applying a pressure difference of 40 cm. water), the results obtained would be significant for present purposes. A good idea would at least be obtained of the pulse pressures, the timing relationship between the arches and the general form of the pulse curves. The apparatus showed promise of giving significant comparative readings for the different arches, which was the prime purpose of the experiments.

In order to be quite sure that differences between the pressures recorded were not due to a lack of absolute uniformity in the two manometers used, the manometers were often reversed in successive experiments. Readings in the systemic arch, for example, were not all taken with the same manometer.

Following each experiment the manometers were simultaneously calibrated against a citrate solution, thus cleaning out the cannulae by flushing them with citrate at the same time. The calibrations were entered in the previous experimental recording.

It was found by a series of tests that the accuracy of measurement was $\pm 0{\cdot}25$ cm. citrate solution pressure (*ca.* 0·2 mm. Hg), and so all measurements were taken to the nearest 0·5 cm. Readings were subsequently converted to their equivalents in mm. Hg.

The toads were prepared in exactly the same way as had been done for the fluorescein experiments, except that anaesthetized toads were always used, because of the difficulty of preventing struggling during the course of the experiments. The position of the needles was adjusted so that they were in corresponding positions in the blood vessels, lying well to the side to prevent any blockage. Tests with the cannulae of the manometers facing in opposite directions in the same artery showed that at the blood velocities involved there was no measurable difference in the pressure recording due to the cannula position. Any slight differences in the angle of the cannulae in the different arches therefore are entirely negligible. The recordings were taken as quickly as possible, because of the danger of the fine needles becoming blocked with clotted blood in spite of the manometers being filled with citrate solution.

B. *Results of pressure measurements experiments*

The basic results, and those upon which most attention is to be focused, were those of comparative readings in the arterial arches (summarized in Table 1). After conversion to mm. Hg the readings appear to be smaller than those obtained by other workers (e.g. Schulz, 1906, *Rana esculenta*, in arterial arches up to 60 mm. Hg). The highest measurements obtained in any of the present experiments were of the order of 45 mm. Hg. It would thus appear that the normal average blood pressure of

Xenopus is lower than that of *Rana* or other Anura. Another immediately striking point is the rather wide range of pressures in different experiments (see lines (*d*), (*e*), (*f*) in Table 1). There appeared to be no relation between the size of animal used and the blood pressure, but a certain percentage of the variations may be due to the extent to which the animals were narcotized.

Table 1. *Pressures in the arterial arches*

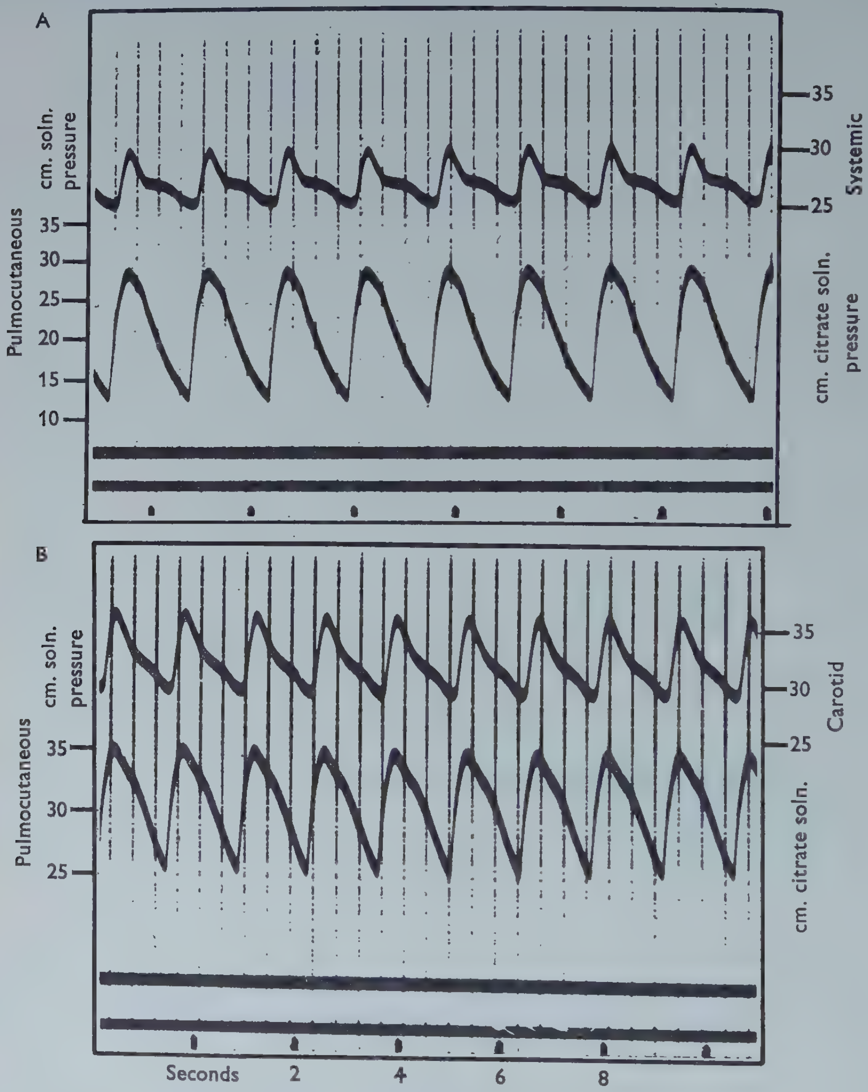
(mm. Hg to the nearest 0·4 mm.)

	Systolic	Diastolic
(A) Simultaneous measurements in the systemic and pulmo-cutaneous arches (averages of 16 records)		
(*a*) Average systemic pressure	25·6	20·4
(*b*) Average pulmo-cutaneous pressure	24·4	13·6
(*c*) Differences between averages	1·2	6·8
(*d*) Range of systemic pressures	17·6–37·6	12·8–30·0
(*e*) Range of pulmo-cutaneous pressures	16·4–34·4	8·4–22·4
(*f*) Range of differences in pressure between arches	0·4– 3·2	3·6–10·0
(B) Simultaneous measurements in the systemic and carotid arches (averages of 11 records)		
(*a*) Average carotid pressure	27·2	22·8
(*b*) Average systemic pressure	27·2	23·2
(*c*) Differences between averages	0	Systemic +0·4
(*d*) Range of carotid pressures	23·6–29·2	19·6–25·2
(*e*) Range of systemic pressures	24·0–29·2	20·4–25·2
(*f*) Range of differences in pressure between arches	Systemic −0·4–+1·2	Systemic −0·4–+1·6
(Standard deviation)	0·23	0·31
(C) Simultaneous measurements in the pulmo-cutaneous and carotid arches (averages of 7 records)		
(*a*) Average carotid pressure	24·4	20·0
(*b*) Average pulmo-cutaneous pressure	23·6	14·0
(*c*) Differences between averages	0·8	6·0
(*d*) Range of carotid pressures	20·8–28·4	18·0–23·2
(*e*) Range of pulmo-cutaneous pressures	20·0–28·0	12·8–16·4
(*f*) Range of differences in pressure between arches	0·4– 1·2	5·2– 6·8

The main features which are brought out by these results are that the pulmo-cutaneous arch pressures are consistently lower than either the carotid or systemic pressures (Text-fig. 5), and that the pressures in the carotid and systemic arches are remarkably similar (Table 1).* The pulmo-cutaneous arches have a pressure at systole about 1 mm. Hg lower and at diastole about 7 mm. Hg lower than the other two arches, and the diastolic pressure differences amount to about one-third of the systemic pressure—quite a considerable amount. These figures are higher than those obtained by Acolat (1938*a*).

* It may be remembered that only simultaneous readings can be used for direct comparison. Since there is so much variation in pressure in different animals and gradual changes may occur during the course of an experiment one cannot, for example, compare the carotid pressure of the lower record with the systemic pressure of the upper record of Text-fig. 5.

Another interesting result of the pressure measurements is the shape of the pulse curves which showed some variation. In general, the pulse curve showed two waves. In the pulmo-cutaneous arch the first wave was by far the major one, the second wave



Text-fig. 5. Records of pressure pulses. A. simultaneous recording of pressure in pulmo-cutaneous and systemic arches. B. simultaneous recording of pressure in pulmo-cutaneous and carotid arches. Horizontal lines: base-lines of manometers.

usually only faintly indicated (Text-fig. 5). In the systemic arch the second wave, although small in the great majority of cases, was somewhat more pronounced (Text-fig. 5). There was no difference between the curves of the carotid and systemic arches.

The first wave of the pulse is due to the main propulsive force of the ventricular contraction, while the secondary curve is caused by the contraction of the bulbus. The cavum aorticum distended to a greater extent on ventricular systole and subsequently contracted more forcibly than the cavum pulmo-cutaneum. Moreover, the more rapid fall of pressure in the pulmo-cutaneous arches tends to reduce the effects of bulbar contraction there. For these reasons the second wave of the pulse is naturally more pronounced in the systemic and carotid arches than in the pulmo-cutaneous arches. It is to be noted particularly that this bulbus contraction does not as a rule play an important part in raising the blood pressure in the arches. The main pressure rise is solely due to the ventricular contraction.

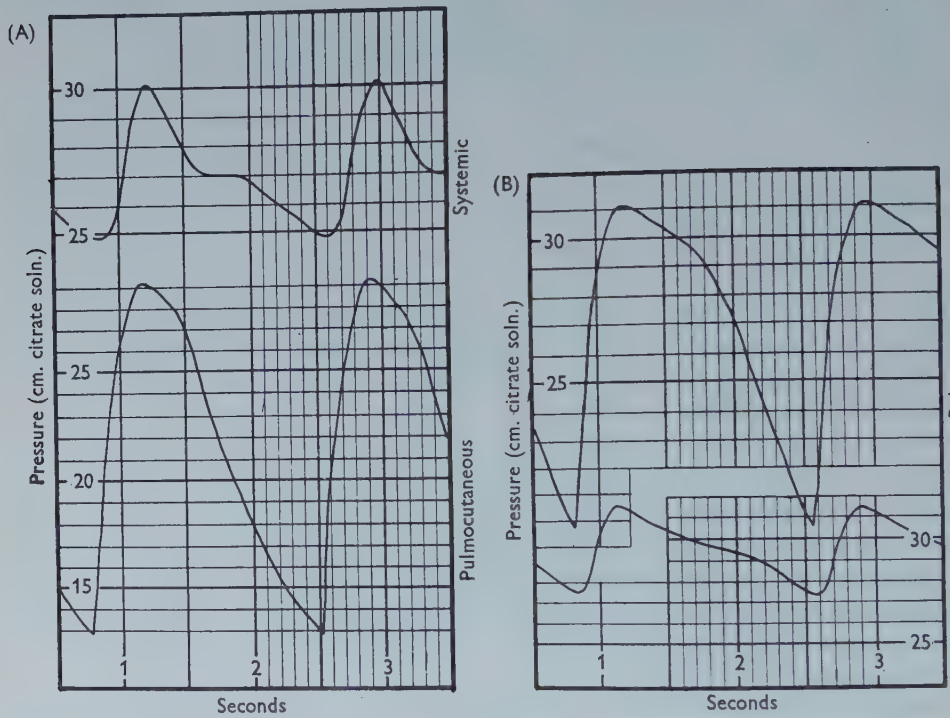
There is no doubt that the features described represent the normal situation. It is true that in a few experiments the second wave of the pulse curve was more marked, in one case it even dominated the picture. However, this phenomenon was always associated with an unusual low blood pressure which could be traced back to rather heavy anaesthesia or some loss of blood. It is, therefore, quite probable that the relatively larger role of the bulbus contraction in those cases can be accounted for by a weaker action of the heart itself. This was confirmed by direct visual observation. In those cases where the ventricular contraction was weak, the bulbus was seen to contract quite markedly, so that the cavum aorticum became almost empty, while in a normally beating heart it did not empty itself completely, and appeared to act more as a pressure relaying mechanism.

The question arises as to whether there is actually a contraction of the muscular wall of the bulbus, or merely an elastic recoil. Histological examination of the bulbus wall reveals no marked elastic fibres, as is the case in the arterial arches. This would seem to indicate that the bulbus is not merely a pressure chamber. Besides, one would not expect an increase in the effect of the bulbar contraction with weakening heart action if the bulbus were merely an elastic chamber. It would appear that the anaesthetic which produced the anomaly has less effect on the bulbar than on the ventricular contraction.

Detailed shape of pulse curves. By projecting the records through an epidiascope and tracing out the curves on paper, it was possible to obtain a more detailed idea of the sequence of events. The curves shown in Text-fig. 6 have been chosen because they exhibit typical characteristics, and also represent successive experiments in which the manometers were reversed. It is evident from a comparison of these curves that there was a slight lag in the time of response of one of the manometers, or more probably a parallax error in the optical system. In Text-fig. 6A the response in the systemic arch and in Text-fig. 6B the response of the pulmo-cutaneous arch are somewhat delayed (same manometer). Nevertheless, the curves show quite clearly what happens. The pulmo-cutaneous pressure rises abruptly and rapidly, whereas there is a slight but true delay in the beginning of the rise in the systemic arch (*ca.* 0·1 sec.). When the latter pressure does increase, it does so more gradually. Both curves (allowing for the time difference in one manometer) reach their maxima at about the same time, although the pulmo-cutaneous pressure at no time becomes equal to that in the systemic arch. The subsequent fall in pressure in the

pulmo-cutaneous arch is invariably more rapid and is more suddenly interrupted by the succeeding ventricular systole.

There cannot be any question of the spiral valve blocking off the entrance to the pulmo-cutaneous arches before the major propulsive force of the ventricular contraction is expended. This is shown by the pulse curves of the pulmo-cutaneous and carotid-systemic arches, which reach their maxima at about the same time. The timing of the bulbus contraction is later, and it can be only then that the spiral valve



Text-fig. 6. Details of pressure pulses. Pressure pulses in systemic and pulmo-cutaneous arches recorded simultaneously. In (B) manometers reversed. Tracings from photographic recordings, enlarged.

may come into contact with the opposite wall of the bulbus. Under these circumstances the significance of the spiral valve is not clear. It might well be that its main function is not to bring about a separation of the blood streams in the way which the 'classical theory' implies, but to ensure, as a ramp, that the carotid and systemic arches receive sufficient blood, in spite of their greater peripheral resistance.

Venous-pressure measurements. A few venous-pressure measurements were taken. These indicated that the pressure in the pulmonary veins was about 3·5 mm. Hg in a lightly anaesthetized toad, while the hepatic vein pressure was only about 1·5 mm. Hg. A small but distinct pulse was visible in the pulmonary vein. By connecting up the manometers simultaneously to the pulmo-cutaneous arch and pulmonary vein it

was possible to interpret the main wave of the pulmonary vein pulse as being due to pressure transmitted through the lung capillary bed. In the hepatic vein, on the other hand, a pulse is either absent or only very faintly indicated.

Simultaneous measurements in systemic arches of opposite sides. Recordings taken of pressures in the systemic arches of opposite sides showed no significant difference in amplitude, shape or timing of the pulse curves.

C. *Discussion of results of pressure measurements*

The results partly explain why the pulmo-cutaneous arches receive more of the blood which leaves the ventricle than the carotid and systemic arches together. There is a pressure in the pulmo-cutaneous arches some 7 mm. Hg less than in the systemic and carotid arches, at the beginning of systole. There is a more rapid drop in pressure in the pulmo-cutaneous arches. There is a distinct pulse in the pulmonary veins. The pressure in the pulmonary veins is higher than in the hepatic veins, illustrating a more direct transmission of pressure through the pulmonary capillary bed. The pulmonary capillaries have a rather large diameter. These facts all clearly indicate that there must be less peripheral resistance to the flow of blood in the pulmonary circuit than through the general circuit. Consequently there is obviously a tendency for the blood to flow into the pulmo-cutaneous arches. Considering the way in which the systemic and carotid arches branch off from the ventral chamber of the truncus, it is hardly surprising that no significant differences appeared between their pulse curves. The lack of difference between the pulse curves of opposite systemic arches is also not surprising. It is true that the right systemic canal branches off from the truncus at a sharper angle, but since the blood flow is not very rapid (as indicated by the fluorescein experiments) this anatomical arrangement could hardly be expected to cause a difference in the pressures of the two sides.

IV. GENERAL DISCUSSION AND CONCLUSION

The present investigations on *Xenopus* have revealed some unexpected results, as neither the 'classical theory' nor the existence of random distribution have been confirmed. There is a kind of selective distribution, but not of the type as required by the 'classical theory'. There is a certain mixing, but not so complete as demanded by random distribution. The main differences from the 'classical theory' concern which side of the heart and which peripheral circuit shows the greater volume flow per unit of time. The difference is partly one of accent, but the accent alters the picture considerably. The situation may be illustrated by the two diagrams of Text-fig. 7. According to the 'classical theory' shown in Text-fig. 7B, the output of the left atrium plus some of the right atrial output supplies the body, and the remainder of right atrial blood goes to lungs and skin. The body circuit would transport more blood than the pulmonary circuit.

The postulate that the lungs of Anura are not able to deal with the same quantity of blood in unit time as the systemic circuit has been advocated for a long time and was emphasized by Acolat (1931*b*). The same idea of a short-circuiting mechanism,

i.e. leakage to the left side to relieve the load upon the lungs, has been stressed in relation to the vertebrate series as a whole. The present results sharply contradict such suggestions, because in *Xenopus* this is obviously not the case.

It is true that the peripheral resistance in the pulmonary circuit is less than in the body circuit, and it is equally true that there is some form of separation in the ventricle. However, although the right atrial blood does not 'contaminate' the left side of the ventricle, the output of the left atrium (being the larger of the two), considerably 'contaminates' the right ventricular blood. The statement of Acolat (1931*b*), that the output of the right atrium is larger, does not hold for *Xenopus*. In *Xenopus* blood from the right side is not given off to the body circulation, but a proportion of left-side blood is conveyed to the pulmonary circulation (Text-fig. 7A). It is the left atrium which supplies *all* arterial branches, whereas the right atrium *merely* supplies the pulmo-cutaneous system.*

There must be a physiological connexion between the right side of the ventricle and the pulmo-cutaneous arch, and between the left side of the ventricle and the other arches. This conclusion is confirmed by a few unusual cases in which the distribution pattern was changed. In one record of injection via the right atrium there was a division in the ventricle lying well to the left of the mid-line. It was notably the only record of that series in which fluorescein appeared simultaneously in all the arterial arches. Thus the right atrial blood has access to the carotid-systemic arches as soon as, and only if, it occupies a proportion of the left side of the ventricle. In two exceptional cases merely the left half of the ventricle coloured after injection via the left side, and only in these cases were the pulmo-cutaneous arches not darker than the others for at least four beats. Apparently the output of the left atrium has access to the body circuit from the left side of the ventricle, but only when it enters the right side of the ventricle does it obtain *full* access to the pulmonary circulation.

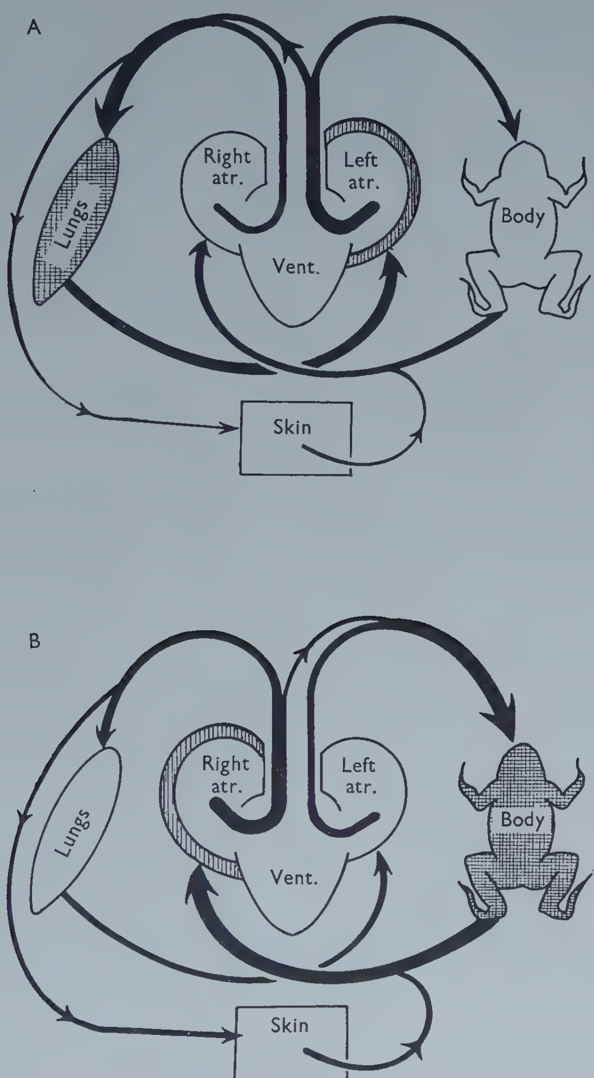
The above-mentioned exceptional cases also show that the ratio of the outputs of the atria may vary, and the outputs are usually less than their anatomical structure leads one to expect. Our observation that the fluorescence in the marginal pockets builds up gradually shows that the atria hold a certain reserve in these pockets. The main bulk of blood is taken in and expelled from the central parts. This is in good accord with observations by Foxon (1951) and Acolat (1938*b*). The atria are in fact acting as reservoirs which are not emptied completely at each beat, and whose degree of emptying may possibly vary from time to time. This, in turn, might change the distribution pattern to some extent.

One of the postulates of the 'classical theory' is the assumption that the carotid labyrinths help to maintain a higher pressure in the carotids than in the systemic arches. This idea has to be discarded, at least for *Xenopus*. Pressures in carotid and systemic arches are equal, hence the carotid labyrinths cannot have such a function.

It is not certain exactly how the bulbus cordis with the spiral valve and its other structural peculiarities contributes to the selective distribution. The experimental

* When discussing basic principles we may ignore the small contamination which must always occur in hearts without complete anatomical division.

records do not show how the blood moves in these regions, apart from the observation that the blood from the right side can pass over the rim of the spiral valve from the cavum aorticum to the cavum pulmo-cutaneum. Other considerations lead to



Text-fig. 7. Diagrammatic representations of blood distribution. (A) *Xenopus*; (B) according to the classical hypothesis.

the conclusion that the spiral valve cannot block off the entrance of the pulmo-cutaneous arches before the bulbus has started its contraction. These facts are not sufficient to explain why the blood entering the body circuit from the left ventricular side does not mix to any extent with the crossing stream of right-side blood going to the pulmonary circulation. This problem therefore remains unsolved at the moment.

If the scheme of Text-fig. 7A applies to other Anura as well, it may explain some of the contractions in the results obtained by other workers. Vandervael (1933) and Foxon (1951) injected only into the pulmonary vein. That they found an apparently random distribution would be as expected, if the distribution were as proposed here. On the other hand, Simons & Michaelis (1953) injected into veins leading to the right atrium and reported a selective distribution in some cases, a result again to be expected according to the present proposals. The scheme may also explain the observations of the authors of the 'classical theory', Gompertz (1884), Ozorio de Almeida (1923) and Acolat (1931*b*), with regard to a physiological division of the ventricle. It is interesting to note that in spite of his disagreement with the 'classical theory', Foxon (1951) does report signs of a physiological division of the ventricle. Foxon (1947) and Foxon & Walls (1947), however, report having made injections via the anterior vena cava with a resulting distribution not unlike the pulmonary vein injections. The scheme will also not explain the results of Noble (1925), or of Savolin (1949).

The light in which the 'classical theory' was considered to be a satisfactory account was largely in the consideration of the oxygen needs of the tissues. By the same sort of consideration, however, Vandervael (1933) draws the conclusion that a random distribution would be more efficient! A re-examination of Vandervael's theoretical objections seems worth while. His argument consists of a deduction of the oxygen distribution in an animal with non-functioning lungs (i.e. during hibernation). If the 'classical theory' applies under these conditions, he argues, there would be a tendency for the body tissues to receive the de-oxygenated blood, while the skin which is now the functional respiratory organ would have the oxygenated blood sent back to it via the pulmo-cutaneous arch and the arteria cutanea magna. The lungs would also be receiving the more highly oxygenated blood. The argument, however, has not been carried far enough. Under these circumstances it is hardly to be expected that the lung tissues would extract for their own metabolism much of the oxygen from the blood sent to them. The large supply of blood in proportion to the small bulk of the lung tissues denies such a supposition. If the circulation is taken a step further, therefore, it is seen that the left atrium would not be receiving de-oxygenated blood, but blood with a fair amount of oxygen. Apart from this, the 'classical theory' proposes that some of the right atrial blood (more highly oxygenated in this case) must pass into the systemic arch in order to balance the differences between the blood capacities of the lungs and the other tissues. This blood, plus the partly oxygenated blood from the left atrium, would then be distributed to the body. Bearing in mind that during hibernation the animal has a much decreased oxygen requirement, Vandervael's argument can be seen to be incorrect. There would be the seemingly undesirable physiological arrangement whereby the main respiratory organ, the skin under these conditions, would be receiving the more highly oxygenated blood through the cutaneous artery, but this does not affect the argument about the oxygen needs of the body.

Nevertheless, these objections against Vandervael's argument do not confirm the 'classical theory', and a random distribution of blood might suffice equally well for

the transportation of oxygen to the tissues. Yet a form of selective distribution *is* present in *Xenopus*. What is its significance in the life of the animal?

The body and the head receive the most highly oxygenated blood, only scarcely contaminated with oxygen-poor blood from the right side. This seems to be a better arrangement for the animal than the conditions proposed by the 'classical theory', according to which mixed blood is supplied to the body. According to the 'classical theory' the lungs receive oxygen-poor blood while according to the present scheme they receive mixed blood. Does this result in a more gradual utilization of the air in the lungs, and if so, has this any advantage for the animal? *Xenopus* may stay under water for considerable lengths of time. During such periods, apparently, skin respiration supplies the body with blood oxygenated to a similarly low degree as proposed by the 'classical theory'.

However, it must be admitted that selective distribution possibly has less meaning in relation to the oxygen needs of the animal than has been advocated. It has been shown (de Graaf, 1957) that the oxygen requirements of *Xenopus* may at times be extremely low. They are able to survive, for example, with most of the haemoglobin of their blood blocked by carbon monoxide. They are also able to survive by cutaneous respiration alone, in spite of the very small size of their cutaneous arteries. It would appear, therefore, that when their lungs are functional, the blood contains more oxygen than is required in many circumstances. We may thus ask whether selective distribution is possibly related to vital needs other than oxygen requirements.

Another point which escapes explanation at the moment is the significance of a hydrodynamic arrangement whereby there is a slow and volumetrically smaller circulation through the general body tissues, while there is a rapid and volumetrically greater flow in the pulmonary circuit. Moreover, a proportion of the blood circulates two or more times through the lungs before being sent to the rest of the body. Is it purely an accident arising from the design of the blood vascular system? This seems unlikely. Pure accidents in physiological design are seldom if ever encountered in nature.

Further research must take a wider form in order to clarify the position. It should include investigations not only into the actual distributional patterns observed, but also into the mechanism whereby it is achieved, into the volume-flow relationships in the different blood vessels and vascular beds, and into the oxygen requirements of the tissues in different seasons.

If the outcome of the present investigations is not proved by further work to be merely an eccentricity of *Xenopus laevis*, it will require a revision of the generally accepted principles of the functioning and physiological design of the blood vascular system of the Anura, and of other vertebrates with imperfectly divided hearts.

V. SUMMARY

1. The structure of the heart of *Xenopus laevis* is described, and the differences between *Xenopus* and *Rana* are stressed.

2. A fluorescein-cinematographic method of tracing blood flow and an optical manometer for the measurement of blood pressure in *Xenopus* are described.

3. The right atrial blood is absorbed into the trabecular meshwork only on the right side of the ventricle.

4. Whereas the output of the right atrium is not, or only in negligible quantities, transferred to the left side of the ventricle, a considerable proportion of the output of the left atrium moves to the right half of the ventricle.

5. The left atrium has a larger output than the right atrium.

6. Almost all the blood expelled from the right atrium is sent to the pulmo-cutaneous arches.

7. The blood from the left atrium is distributed to all the arterial arches and the pulmo-cutaneous arches receive a considerable proportion of this blood.

8. More blood flows through the pulmo-cutaneous arches at each beat than is sent through the carotid and systemic arches together.

9. The rate of flow in the pulmonary circuit is much higher than that in the body circuit.

10. A physiological connexion is demonstrated between the left side of the ventricle and the systemic and carotid arches, and between the right side of the ventricle and the pulmo-cutaneous arches.

11. Pressures in the pulmo-cutaneous arch are consistently lower than in either the carotid or systemic arches.

12. The pressures in the carotid and systemic arches are remarkably similar. There is, therefore, no sound reason for postulating a mechanism in the carotid labyrinths which should maintain a higher pressure in the carotids than in the systemics.

13. The pulse curves in the arches show two waves: the first, major, one produced by contraction of the ventricle, the second one by contraction of the bulbus cordis.

14. The spiral valve may not come into contact with the opposite wall of the bulbus before contraction of the latter. Before that time, i.e. as long as the major propulsive force of the ventricular contraction is not expended, the cavum pulmo-cutaneum is in open communication with the ventricle.

15. The pulmonary veins show a weak pulse, and their pressure is higher than in the hepatic veins. This indicates smaller resistance in the pulmonary circuit than in the body circuit.

16. The selective distribution is neither in agreement with the 'classical theory' nor with ideas of random distribution.

17. The forces underlying the selective distribution and the significance of the pattern are discussed.

I must express my gratitude and indebtedness to: Prof. B. J. Krijgsman, under whose supervision the work was carried out, for constant encouragement and advice; Prof. J. H. Day, for his interest in the project and for a number of suggestions; Prof. R. Goetz, of the Surgical Research Department, for the loan of the photo-kymograph and other assistance; Mr R. L. Liversidge, for the use of the cinematographic equipment and for much help in the construction of experimental apparatus; the Technical Staff of the Physics Department for the construction of the optical manometers and for other services; Dr N. Millard, for the loan of a microscope projection lamp; and Mr T. Stafford-Smith, for the loan of a parallax adjustor for the ciné camera.

REFERENCES

Acolat, L. (1931*a*). Recherches anatomiques relatives à la séparation du sang veineux et du sang artériel dans le cœur de la Grenouille. *C.R. Acad. Sci., Paris*, **192**, 767.

Acolat, L. (1931*b*). Recherches physiologiques relatives à la séparation du sang veineux et du sang artériel dans le cœur de la Grenouille. *C.R. Acad. Sci., Paris*, **192**, 849.

Acolat, L. (1938*a*). Étude comparée de la pression sanguine dans le circuit pulmonaire et dans le circuit général chez les Batraciens et les Reptiles. *C.R. Acad. Sci., Paris*, **206**, 207.

Acolat, L. (1938*b*). Sur la répartition du sang artériel dans les artères centrales des Batraciens et des Reptiles. *C.R. Acad. Sci., Paris*, **206**, 940.

Brücke, E. (1852). Beiträge zur vergleichenden Anatomie und Physiologie des Gefäßsystems. I. Über die Mechanik des Kreislaufes bei den Amphibien. *Denkschr. Akad. Wiss. Wien*, Math.-naturw. Kl., **3**. (Ref. in H. Winterstein, *Handb. vergl. Physiol.* **1**, 1, 1925.)

de Graaf, A. R. (1957). A note on the oxygen requirements of *Xenopus laevis*. *J. Exp. Biol.* (in the Press).

Foxon, G. E. H. (1947). Mode of action of the heart of the frog. *Proc. Zool. Soc. Lond.* **116**, 565–71.

Foxon, G. E. H. (1948). A simple apparatus for making rapid serial radiographs of small objects. *Brit. J. Radiol.* **21**, N.S., 301–4.

Foxon, G. E. H. (1951). A radiographic study of the passage of the blood through the heart of the frog and toad. *Proc. Zool. Soc. Lond.* **121**, 529–38.

Foxon, G. E. H. (1953). A note on Simons' and Michaelis' article. *Nature, Lond.*, **171**, 801.

Foxon, G. E. H. (1955). Problems of the double circulation in vertebrates. *Biol. Rev.* **30**, 195–228.

Foxon, G. E. H. & Walls, E. W. (1947). The radiographic demonstration of the mode of action of the heart of the frog. *J. Anat., Lond.*, **81**, 111–17.

Fritsch, G. (1869). Zur vergleichenden Anatomie des Amphibienherzens. *Arch. Anat. Physiol.* p. 654. (Ref. in J. Winterstein, *Handb. vergl. Physiol.* **1**, 1, 1925).

Gaupp, E. (1899). *Anatomie des Frosches*, vols. **2** and **3**. Brunswick.

Gompertz, G. (1884). Über Herz und Blutkreislauf bei nackten Amphibien. *Arch. Anat. Physiol.* p. 242.

Gregg, D. E., Eckstein, R. W. & Fineberg, M. A. (1937). Pressure pulses and blood-pressure values in unanaesthetized dogs. *Amer. J. Physiol.* **118**, 399.

Hamilton, W. F. (1946). In Howell's *Text book of Physiology*. London.

Hamilton, W. F., Brewer, G. & Brotman, I. (1934). Pressure pulse contours in the intact animal. I. Analytical description of a new high-frequency hypodermic manometer with illustrative curves of simultaneous arterial and intracardiac pressures. *Amer. J. Physiol.* **107**, 427.

Hazelhoff, E. H. (1952). Die Trennung der Blutmassen mit verschiedenem Sauerstoffgehalt im Froschherzen. *Experientia*, **8**, 471.

Millard, N. (1941). The vascular anatomy of *Xenopus laevis*. *Trans. Roy. Soc. S. Afr.* **28**, 387–439.

Millard, N. (1942). Abnormalities and variations in the vascular system of *Xenopus laevis*. *Trans. Roy. Soc. S. Afr.* **29**, 9–28.

Millard, N. (1945). The development of the arterial system of *Xenopus laevis*, including experiments on the destruction of the larval aortic arches. *Trans. Roy. Soc. S. Afr.* **30**, 217–34.

Millard, N. (1949). The development of the venous system of *Xenopus laevis*. *Trans. Roy. Soc. S. Afr.* **32**, 55–99.

Noble, G. K. (1925). The integumentary, pulmonary and cardiac modifications correlated with increased cutaneous respiration in the Amphibia: A solution of the 'Hairy Frog' problem. *J. Morph.* **40**, 341–416.

OZORIO DE ALMEIDA, M. (1923). Sur la séparation des ondes artérielle et veineuse dans l'appareil circulatoire de la Grenouille. *C.R. Soc. Biol., Paris*, **89**, 1019–21.
RAO, C. R. N. & RAMANNA, B. S. (1925). Notes on the conus arteriosus of some genera of Engystomatidae and certain examples of *Rana* and *Bufo*. *Proc. Zool. Soc. Lond.* **2**, 1445–78.
SABATIER, A. (1873). *Études sur le cœur et la circulation centrale dans la série des Vertébrés.* Montpellier et Paris. (Ref. in Winterstein, *Handb. vergl. Physiol.* **1**, 1, 1925.)
SAVOLIN, K. (1949). A study of the distribution of oxygenated and deoxygenated blood through the conus arteriosus of the toad (*Bufo bufo* L.). *Soc. Sci. Fenn., Comm. Biol. Helsingf.* **10**, 1–12.
SCHULZ, F. N. (1906). Studien über das Verhalten des Blutdruckes von *Rana esculenta* unter den verschiedenen äusseren Bedingungen, insbesondere bei verschiedener Körpertemperatur. *Pflug. Arch. ges. Physiol.* **115**, 386.
SIMONS, J. R. & MICHAELIS, A. R. (1953). A cinematographic technique using ultra-violet illumination, for Amphibian blood circulation. *Nature, Lond.*, **171**, 801.
VANDERVAEL, F. (1933). Recherches sur le mécanisme de la circulation du sang dans le cœur des Amphibiens anoures. *Arch. Biol., Paris*, **44**, 571–606.
WIGGERS, C. J. (1928). *The Pressure Pulses in the Cardiovascular System.* London.

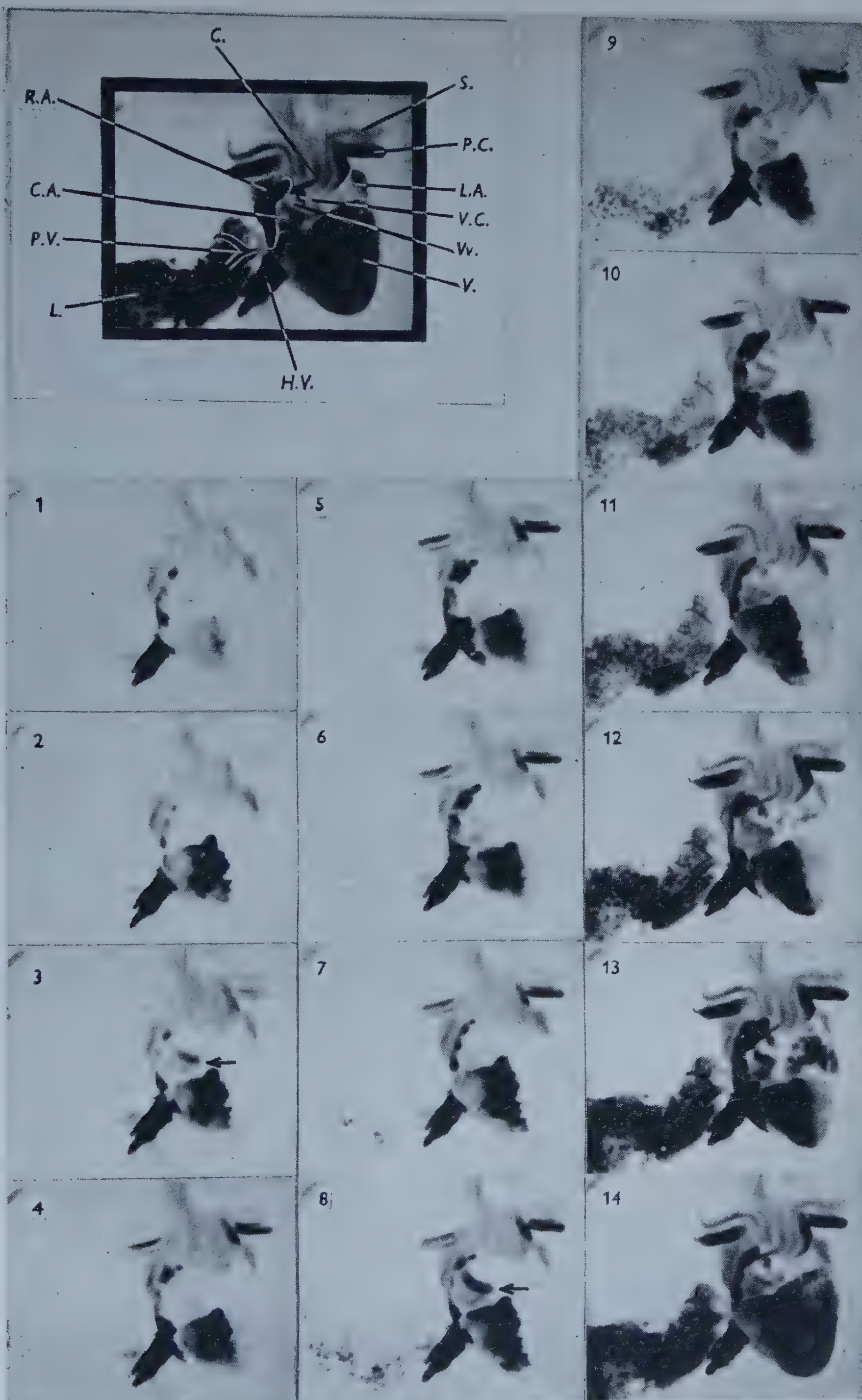
EXPLANATION OF PLATES

PLATE 3

Male *Xenopus*. No anaesthetic administered. Heart rate 40/min. 0·025 ml. 1 % fluorescein–Ringer injected into a hepatic vein. Filming: 16/sec. at *f* 3·5. Three subsequent heart beats are shown. About every fifth frame printed. Ventricular systole starts at 3, 9, 12. Ventricular diastole starts at 1, 6, 11, 14. *C.* carotid arches; *C.A.* bulbus cordis; *H.V.* hepatic vein; *L.* lung; *L.A.* left atrium; *P.C.* pulmo-cutaneous arch; *P.V.* pulmonary vein; *R.A.* right atrium; *S.* systemic arch; *V.* ventricle; *V.C.* ventral chamber of truncus; *Vv.* valves IA and III (see Text-fig. 2, p. 146, valves at distal end of bulbus).

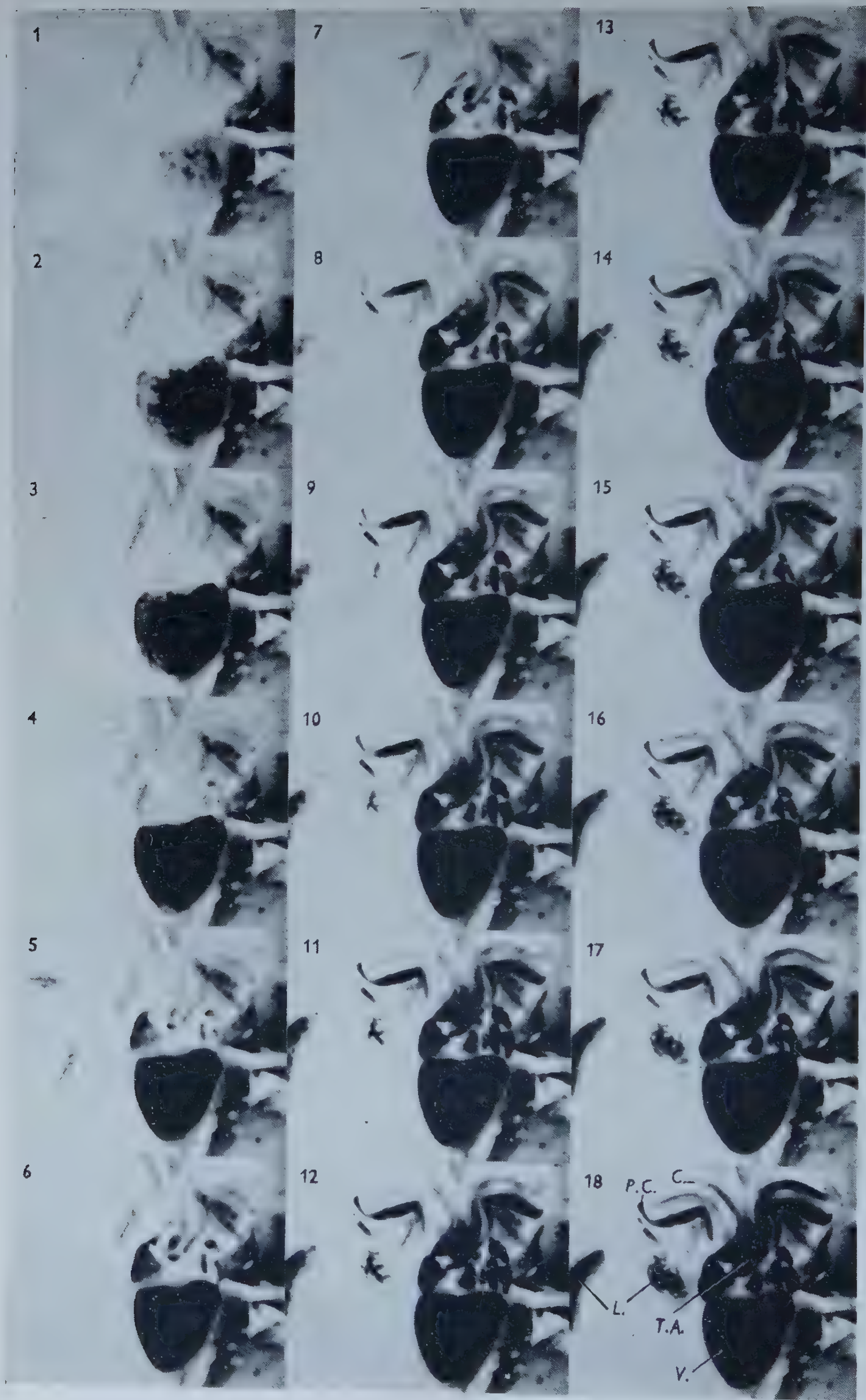
PLATE 4

Male *Xenopus*. 0·6 ml. urethane as anaesthetic. Heart rate 45/min. 0·12 ml. 1 % fluorescein–Ringer injected into a pulmonary vein. Filming: 8/sec. at *f* 4·0. Two subsequent heart beats are shown. Every fourth frame printed. Ventricular systole starts at 4 and 16, ventricular diastole starts at 1 and 13.



De GRAAF—INVESTIGATIONS INTO THE DISTRIBUTION OF BLOOD IN THE HEART AND AORTIC ARCHES OF *XENOPUS LAEVIS* (DAUD.)

(*Facing p.* 172)



De GRAAF—INVESTIGATIONS INTO THE DISTRIBUTION OF BLOOD IN THE HEART AND AORTIC ARCHES OF *XENOPUS LAEVIS* (DAUD).

A NOTE ON THE OXYGEN REQUIREMENTS OF *XENOPUS LAEVIS*

By the late A. R. de GRAAF, M.Sc.*
Department of Zoology, University of Cape Town

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INTRODUCTION

While experimenting on the blood distribution in *Xenopus* (de Graaf, 1957) the desirability of obtaining some idea of the oxygen requirements of the animal became more and more apparent. Quite unexpectedly an ideal starting point was encountered in the form of an abnormal specimen which appeared to have no red blood pigment at all. The specimen was one of a regular supply kept in the Department of Zoology and had probably been kept in captivity for some months.

It had been injected with 0·6 ml. 20% ethyl-urethane as an anaesthetic, and the abdominal cavity had been opened for experiment before anything untoward was noticed. The toad struggled in a normal fashion while the anaesthetic was being injected. External features appeared to be quite normal except for a slightly more bluish tinge to the skin than usual. After opening the abdomen it was noticed that the muscles had a bluish tinge, and the blood vessels were colourless apart from the usual chromatophores in their walls. The ventricle was whitish in colour, the atria pale and translucent, the main arterial arches also pale and somewhat translucent, and the spleen was white with a very slight yellowish tinge. The general anatomical structure was quite normal, although the spleen was somewhat smaller than usual. The specimen was a partially mature female.

About 1 ml. of blood was removed and oxalated. It was quite colourless to the eye. Fresh blood smears revealed no cellular elements apart from some bodies which had the appearance of spindle cells. Unfortunately, it has not been possible either to have a spectroscopic examination or an iron estimation performed on the blood sample.

The toad was preserved in 5% formalin for some days and the spleen was subsequently removed, sectioned and stained. Comparison of the sections with those of a normal *Xenopus* spleen prepared in the same way revealed that there were no normal mature erythrocytes, although the other blood elements and the reticular structure of the spleen looked normal. Mr Jacobson of the Medical School kindly examined the preparation and found normoblasts present, although these did not show the cytoplasmic staining typical of mature erythrocytes.

Wolvekamp & Lodewijks (1934) noted that the number of erythrocytes in *Rana* tended to drop markedly when they were kept in captivity. They accounted for this by the greatly diminished activity and metabolism of the frog. No reports have

* Edited by the Department after the death of Mr de Graaf.

been found, however, of the red blood count decreasing to as low a level as seen in this *Xenopus* specimen. The apparently normal behaviour of this animal prompted a brief investigation of oxygen supply in relation to survival.

EXPERIMENTAL

In the first series of experiments an attempt was made to block the haemoglobin with carbon monoxide. Fifteen apparently normal toads were placed in a glass jar into which illuminating gas was tapped for 5 min. After a short period of 1 or 2 min. for air to circulate, some of the toads were removed and placed in separate containers free from gas. With the other animals the gas application was repeated a number of times, up to a total exposure of 30 min. Immediately after the experiment blood was removed from one of the specimens and tested for carbon monoxide content by the colorimetric pyrogallic-tannic acid method (Kolmer & Boerner, 1945). This showed that after a 30 min. exposure to gas 80–100% of the haemoglobin was blocked by carbon monoxide. Nevertheless, all the toads survived the 30 min. exposure, although it took them some hours to revive from the semi-comatose state produced by the treatment. Subsequent inspection during 4 days did not show any ill effects whatsoever, although the haemoglobin had by no means been completely regenerated by the end of that time. It appears safe to draw the conclusion that under certain circumstances *Xenopus* can withstand a nearly complete elimination of its haemoglobin. It is not suggested, however, that the toad will be able to manage without haemoglobin at periods of great activity, for example, the breeding season.

Another series of experiments was carried out to obtain some information on the significance of cutaneous respiration. A number of animals were placed in a container and prevented from reaching the surface of the water by wire gauze. Tap water of an average temperature of 15° C. was then kept flowing through the container at a slow rate. The oxygen content of the inflowing water was just under 5 ml./l. No food was given to the animals. It was found that the animals were still alive after a period of 4 weeks, when the experiment was discontinued. Apparently they did not suffer any obvious discomfort. In fact, they did not lie motionless all the time, but swam about occasionally and also became active when disturbed. These experiments indicate that in spite of the very small size of the arteriae cutaneae magnae (cross-sectional area about one-ninth of that of the pulmonary artery) cutaneous respiration is well able to support the animals under the prevailing conditions.

DISCUSSION

The occurrence, even as an abnormality, of a vertebrate without haemoglobin is almost unprecedented, except for the leptocephalus larvae of eels, and certain sluggish South Georgian fishes, which possess neither erythrocytes nor blood pigments (Ruud, 1954). The absence of haemoglobin in an otherwise apparently normal toad, therefore, opens interesting speculations regarding the significance of haemoglobin in poikilothermic animals. Barcroft (1928) has said that as one passes

from warm-blooded to cold-blooded vertebrates, quite a number of factors tend to make the demand for oxygen more nearly commensurate with the supply which can be maintained by a blood devoid of pigment. Amongst these factors he notes that the demand for oxygen is less, and that the oxygen is more soluble in plasma at lower temperatures. Barcroft estimates that from the points of supply and demand of oxygen, a frog without haemoglobin would be some two hundred times better off than a mouse in the same circumstances. Although purely speculative, this paints rather a vivid picture. His opinion is well in agreement with the experiments of Nicloux (1923 *a*, *b*), who reported that carps, pikes and eels survive for at least 4 hr., apparently without trouble, when the haemoglobin is blocked to the extent of some 90% by carbon monoxide. Gréhant (1887) stated that *Rana* survived for at least 3 days in a gas mixture containing 50% carbon monoxide.

Wolvekamp (1932) and Wolvekamp & Lodewijks (1934) have determined the oxygen dissociation curves of the blood of *Rana esculenta*. Since the tension of carbon dioxide in the lungs varies between 7 and 14 mm. Hg, since the carbon dioxide tensions in the circulatory system and in the tissues are very probably not higher than 14 mm. Hg, (Campbell, 1924, 1926), and since the oxygen tension in the tissues is not usually below 28 mm. Hg, it follows from these dissociation curves that the haemoglobin does not deliver a large quantity of oxygen to the tissues. Most of the oxygen is kept in store by the blood. The blood might act, to a considerable extent, as an emergency mechanism for times of greater activity and increased metabolism.

Rana hibernates for long periods at low temperatures under conditions where only cutaneous respiration is possible. It has been shown by Serfaty & Gueutal (1943) that *R. esculenta* is able to survive for 2–3 weeks when totally submerged in well-oxygenated water at a temperature as high as 14–15° C. Charles (1931) performed experiments which indicate that the cutaneous respiration of *Xenopus* accounts for about one-quarter to one-third of the total absorption of oxygen in richly oxygenated surroundings. Similar estimations by Krogh (1904) show a greater cutaneous respiration (one-third to one-half) in *Rana esculenta* and *R. fusca*. The difference between the genera can probably be explained by the relative sizes of the arteriae cutaneae magnae, those of *Rana* being much larger.

The present experimental results agree well with the findings of other workers and support the contention that the oxygen requirements of *Rana* and *Xenopus* may fall to a very low level at certain periods, although it is not claimed that such conditions always prevail in nature. Nevertheless, the question must be faced as to whether the type of selective distribution of blood advocated by the 'classical theory', or the kind of distribution found in *Xenopus* (de Graaf, 1957) is *really* related to the oxygen needs of the tissues. One wonders if the starting point of all considerations on blood distribution in Amphibia, that is, the oxygen need of head and body, is not altogether wrong. The possibility that selective distribution serves some quite different purpose must be seriously considered. This 'different purpose', however, is completely unknown at the moment and future research will have to clarify the position.

SUMMARY

1. It is shown that survival in *Xenopus* is not dependent upon the presence in the blood of a functional respiratory pigment.

2. The implications of this finding are discussed in relation to the possibility of selective distribution of the blood to different parts of the body.

REFERENCES

BARCROFT, J. (1928). *The Respiratory Function of the Blood.* Vol. **2**. *Haemoglobin.* Cambridge University Press.

CAMPBELL, J. A. (1924). Changes in the tensions of CO_2 and O_2 in gases injected under the skin and into the abdominal cavity. *J. Physiol.* **59**, 1–16.

CAMPBELL, J. A. (1926). The normal CO_2- and O_2-tensions in the tissues of various animals. *J. Physiol.* **61**, 248–54.

CHARLES, E. (1931). Metabolic changes associated with pigmentary effector activity and pituitary removal in *Xenopus laevis*. I. Respiratory exchange. *Proc. Roy. Soc.* B, **107**, 486–503.

DE GRAAF, A. R. (1957). Investigations into the distribution of blood in the heart and aortic arches of *Xenopus laevis*. *J. Exp. Biol.* (in the Press).

GRÉHANT (1887). *C.R. Soc. Biol., Paris*, **39**, 198 (cited by Nicloux, 1923*b*).

KOLMER, J. A. & BOERNER, F. (1945). *Approved Laboratory Technic.* New York.

KROGH, A. (1904). On the cutaneous and pulmonary respiration of the frog. *Skand. Arch. Physiol.* **15**, 328.

NICLOUX, M. (1923*a*). Action de l'oxyde de carbone sur les poissons et capacité respiration du sang de ces animaux. *C.R. Soc. Biol., Paris*, **89**, 1328–31.

NICLOUX, M. (1923*b*). Considérations générales sur l'intoxication oxycarbonique et la quantité minima d'oxygène nécessaire dans le sang pour assurer la vie; leur intérêt thérapeutique. *C.R. Soc. Biol., Paris*, **89**, 1331–4.

RUUD, J. T. (1954). Vertebrates without erythrocytes and blood pigment. *Nature, Lond.*, **173**, 847–50.

SERFATY, A. & GUEUTAL, J. (1943). La résistence de la grenouille à l'asphyxie lors d'une immersion prolongée. *C.R. Soc. Biol., Paris*, **137**, 154–6.

WOLVEKAMP, H. P. (1932). Untersuchungen über den Sauerstofftransport durch Blutpigmente bei *Helix, Rana* und *Planorbis*. *Z. vergl. Physiol.* **16**, 1–38.

WOLVEKAMP, H. P. & LODEWIJKS, J. M. (1934). Uber die Sauerstoffbindung durch Hämoglobin vom Frosch. *Z. vergl. Physiol.* **20**, 382–7.

THE RELATIONSHIP BETWEEN THE DIURNAL LIGHT CYCLE AND THE TIME OF OVULATION IN MICE

A. W. H. BRADEN*
Institute of Animal Genetics, West Mains Road, Edinburgh

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INTRODUCTION

It is now well established that in rats and mice the events of the oestrous cycle are closely linked with the diurnal rhythm of light and darkness. If the times of light and darkness are reversed a corresponding change in the times of oestrus and ovulation occurs (Hemmingsen & Krarup, 1937; Browman, 1937; Snell, Fekete, Hummel & Law, 1940; Snell, Hummel & Abelmann, 1944; Austin & Braden, 1954; Braden & Austin, 1954). There is good evidence that the effect of the diurnal light cycle on the times of oestrus and ovulation is mediated by the eyes, the central nervous system and the anterior pituitary gland (Hill & Parkes, 1933; Bissonette, 1938; Clark, McKeown & Zuckerman, 1939; Everett, Sawyer & Markee, 1949; and others).

The experiments to be described in this paper were designed to investigate the effect on the time of ovulation of changes in the relative lengths of the light and dark phases of the 24 hr. cycle. These changes occur during the course of the year in non-equatorial latitudes such as that of Edinburgh (latitude 56° N), where studies were made on mice kept under natural lighting conditions. Investigations were also made on mice transferred to a room in which the times of light and dark were reversed. Differences found between three outbred stocks of mice have indicated that response to the diurnal light cycle by the mouse is probably determined to some extent at least by the genetic constitution of the animals.

MATERIALS AND METHODS

In the first experiment (December 1954), female mice from about eight out-bred strains were used and no separate record was kept of the results from each strain, but in subsequent experiments random-bred females from the stocks L, C and PCT were used, and the results from each strain recorded separately. The L stock was that employed by Dr N. Bateman in this laboratory for genetical studies on lactation, the C stock was that used by Dr D. S. Falconer for studies of the inheritance of body size, and the PCT stock was obtained from Dr T. C. Carter.

In the experiments carried out under the natural diurnal light cycle, one male was added to each cage of three to five females at 5–6 p.m. and the females were inspected for the presence of vaginal plugs during the 20 min. preceding 10 p.m., 12 midnight, 2 a.m., 4 a.m., 6 a.m., 8 a.m., 10 a.m. and 2 p.m. (All the times

* Present address: C.S.I.R.O., Sheep Biology Laboratory, Prospect, N.S.W., Australia.

quoted are Greenwich Mean Time (G.M.T.).) When a mated female was found in any cage, the male was replaced by a fresh male. Males of L strain were used throughout the experiments. Mated females were killed at each of the above-mentioned hours, and the eggs that had been shed were recovered from the Fallopian tubes by dissection under physiological saline solution. The eggs were examined in the fresh state by phase-contrast microscopy. When no eggs could be found in the Fallopian tubes, the ovaries were inspected to ensure that ripe follicles were present, and often the eggs obtained by puncturing these follicles were examined by phase-contrast microscopy. The arrival of the eggs in the distended portion of the Fallopian tube has been taken as synchronous with ovulation; the time required for the egg to travel from the ovary to the distended portion of the ampulla—the site of fertilization—is almost certainly relatively short.

Studies of the time relations of ovulation in mice maintained under a reversed diurnal light cycle were also made. The mice were kept in a room in which the sole source of light was an 80 W. fluorescent strip-lamp which was switched on and off at certain set times by means of an electric clock relay. Two 24 hr. cycles were used: in the first the dark phase was of 10 hr. duration (5 a.m. to 3 p.m.) and in the second it was of 4 hr. duration (8 a.m. to 12 noon). The females were subjected to an acclimatization period of 3–5 weeks at the beginning of each experiment. A male was then introduced into each cage of three to five females at 8.30 a.m. and females that were found to have vaginal plugs were killed at either 9 a.m., 10 a.m., 11 a.m., 12 noon, 1 p.m., 2 p.m., 3 p.m., 4 p.m., 5 p.m., 6 p.m., or 8 p.m. The occurrence of ovulation was ascertained as described above.

The results were analysed by the probit method. The abscissae (times in hours) were not transformed.

RESULTS

Natural diurnal light cycles. In the December 1954 experiment, a total of 137 females of mixed origin was used. Eight mice found to have mated before 5 p.m. were killed at 5 p.m., but ovulation had not begun (Table 1). The next time of killing was 10 p.m. when one out of eleven mice was found to have ovulated. There-

Table 1. *The progress of ovulation with time in a mixed group of female mice kept under natural lighting conditions and examined in December* 1954

Time of day	No. of mice			Mean no. of eggs per mouse ovulated
	Examined	With tubal eggs	Percentage	
5 p.m.	8	0	0	—
10 p.m.	11	1	9·1	10·0
12 midnight	10	4	40	9·3
2 a.m.	20	8	40	7·7
4 a.m.	22	13	59·1	9·9
6 a.m.	24	18	75	9·4
8 a.m.	14	9	64·3	13·0
10 a.m.	14	13	92·9	10·2
2 p.m.	14	14	100	11·6

after, the percentage of mice that had ovulated gradually increased until 100% was reached between 10 a.m. and 2 p.m. (see Fig. 1). In the mice as a group, therefore, ovulation took place over a period of 14–16 hr. It should be remarked that, as coitus and ovulation proceeded to some extent concurrently in the population (the mean interval between the two events was 5 hr.), the mice killed at the earlier hours (i.e. 10 p.m. and 12 midnight) could not have been an entirely representative sample of the population, for they would have necessarily included only the mice that had mated before the time of killing. If, as is likely, the mice that mate early are the ones that ovulate early in the period, then the results obtained at 10 p.m. and 12 midnight would probably have been a little too high and those obtained at later hours perhaps a little low. However, as this bias was common to all the sets of data obtained, it can be neglected in comparisons between the results recorded under different light cycles or for different strains.

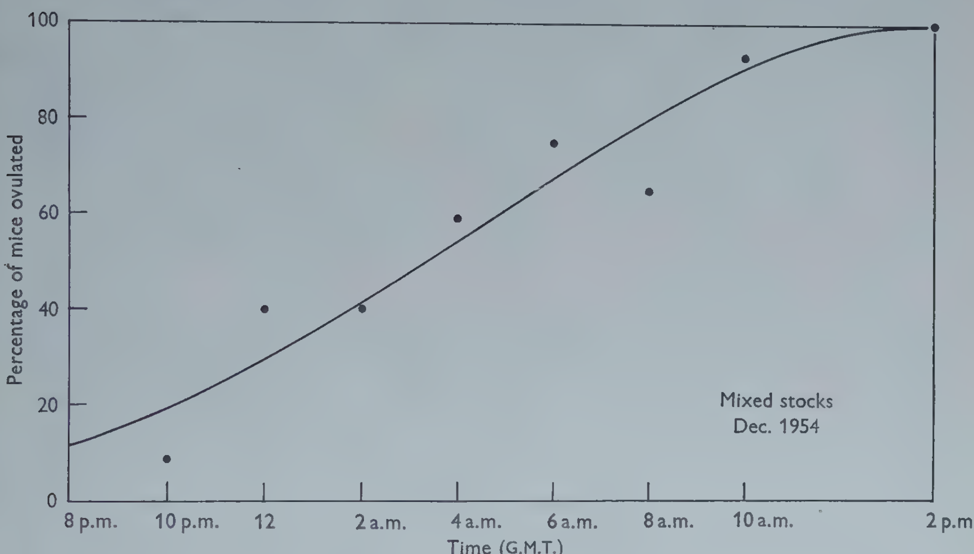

Fig. 1. The progressive increase in the percentage of mice ovulated. December 1954 experiment. The mice were from a number of random-bred stocks and were kept under natural lighting conditions.

In June 1955 the time of ovulation was studied in females of L and C stocks. Ovulation began in L stock females at about 11 p.m. and was complete by about 8 a.m.—a total period of approximately 9 hr. The detailed results are set out in Table 2 and illustrated graphically in Fig. 2. By probit analysis of these results the slope of the probit line relating the transformed percentage of mice ovulated with time was found to be $0{\cdot}42 \pm 0{\cdot}07$. It was estimated that the time by which 50% of the mice would have ovulated was 4·16 a.m. ± 7 min. In the group of females from C stock ovulation began at about 12 midnight and was complete by about 9 a.m. (see Table 2 and Fig. 3). Ovulation thus required a period of 9 hr. in this group. The slope of the probit line was $0{\cdot}405 \pm 0{\cdot}079$, and the time by which 50%

of the mice would have ovulated was 5.00 a.m. ± 6 min. The slopes of the probit lines for the two groups of mice clearly did not differ significantly, but there was a significant ($\chi_1^2 = 52{\cdot}84$; $P < 0{\cdot}001$) difference between the mid-points of the ovulation periods for the two stocks.

Table 2. *The increase in the number of mice ovulated with time in L and C stock females kept under natural lighting conditions and examined in June* 1955

Time of day	L stock				C stock			
	No. of mice			Percentage of mice that had tubal eggs	No. of mice			Percentage of mice that had tubal eggs
	Ex-amined	With tubal eggs	With ≥8 tubal eggs		Ex-amined	With tubal eggs	With ≥7 tubal eggs	
10 p.m.	6	0	0	0	—	—	—	—
12 midnight	12	1	0	8·3	7	0	0	0
2 a.m.	20	3	0	15	10	3	0	30
4 a.m.	12	6	5	50	10	3	3	30
6 a.m.	12	7	7	58·3	9	5	5	55·6
8 a.m.	20	20	20	100	20	18	16	90
10 a.m.	12	12	12	100	10	10	10	100

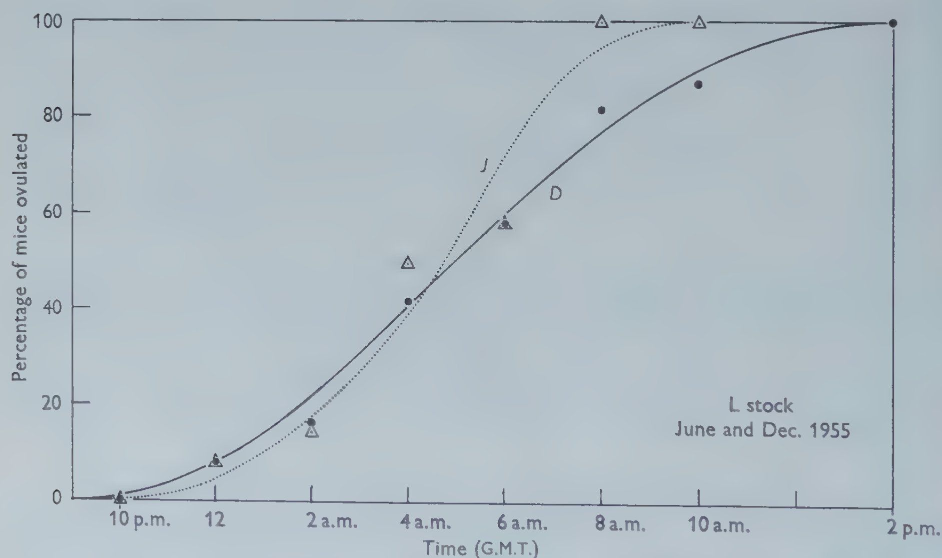

Fig. 2. Illustrating the difference in the periods required for ovulation in groups of L stock mice in June (curve *J*) and December (curve *D*). Mice kept under natural lighting conditions.

In December 1955 ovulation began in a group of ninety-nine females from L stock at about 11 p.m., but was not complete until about midday—a total period of about 13 hr. (see Fig. 2). The detailed results are given in Table 3. In thirty-eight C stock females killed at 10 p.m., 2 p.m., 6 a.m., and 10 a.m., ovulation had begun before 10 p.m. and was not quite complete at 10 a.m. the next morning (see Table 3 and

Fig. 3). The period required for ovulation in the C stock population in December may be estimated as 13–14 hr. The slopes of the probit lines for the two groups were $0{\cdot}286 \pm 0{\cdot}048$ (L stock) and $0{\cdot}235 \pm 0{\cdot}067$ (C stock). The mid-points of the ovulation periods were 5.11 a.m. ± 9 min. and 2.54 a.m. ± 71 min., respectively. Neither the slopes nor the mid-points differed significantly ($\chi_1^2 = 2{\cdot}72$, $P < 0{\cdot}1$, for the mid-points).

Table 3. *The increase in the number of mice ovulated with time in L and C stock females kept under natural lighting conditions and examined in December* 1955

Time of day	L stock				C stock			
	No. of mice			Percentage of mice that had tubal eggs	No. of mice			Percentage of mice that had tubal eggs
	Examined	With tubal eggs	With ≥8 tubal eggs		Examined	With tubal eggs	With ≥7 tubal eggs	
10 p.m.	12	0	0	0	6	1	0	16·7
12 midnight	12	1	1	8·3	—	—	—	—
2 a.m.	12	2	0	16·7	10	3	2	30·0
4 a.m.	12	5	4	41·7	—	—	—	—
6 a.m.	12	7	7	58·3	10	9	8	90·0
8 a.m.	12	10	10	81·3	—	—	—	—
10 a.m.	15	13	12	86·7	12	11	11	91·7
2 p.m.	12	12	12	100	—	—	—	—

In both the June and December (1955) experiments records were kept of the number of females that copulated before 10 p.m., between 10 p.m. and midnight, between midnight and 2 a.m., etc. The results from L and C strains were combined. In the June experiment 63 % of the females were found to have vaginal plugs before midnight, 75 % before 2 a.m. and 98 % before 8 a.m. From the graphs shown in Figs. 2 and 3 it may be estimated that 63 % of the mice would have ovulated by 6 a.m. and 75 % by 6.45 a.m., so that the mean mating-ovulation interval may be stated as approximately 5 hr. In the December experiment 30 % of the females had copulated before 10 p.m., 60 % had copulated before midnight, 75 % had copulated before 2 a.m., and 95 % had copulated before 6 a.m. By comparing these results with the ovulation curves for L and C stock females in December (Figs. 3, 4), the mean mating-ovulation interval was again estimated as about 5 hr. Snell *et al.* (1940) considered that the mean interval between mating and ovulation was probably not in excess of 2–3 hr. The difference between their conclusion and the present finding may be related to a difference between the strains employed or to the small number of animals used by Snell. The magnitude of the interval between coitus and ovulation will depend to some extent on the experimental conditions. For instance, if a male has been used on a number of consecutive nights, it may not copulate with a female until several hours after the onset of oestrus.

Reversed diurnal light cycles. In females of the PCT stock kept under the 10 hr. dark/14 hr. light cycle, ovulation began before 9 a.m. (probably between 7 a.m. and 8 a.m.) and was complete by 5 p.m. (Table 4). The total period over which

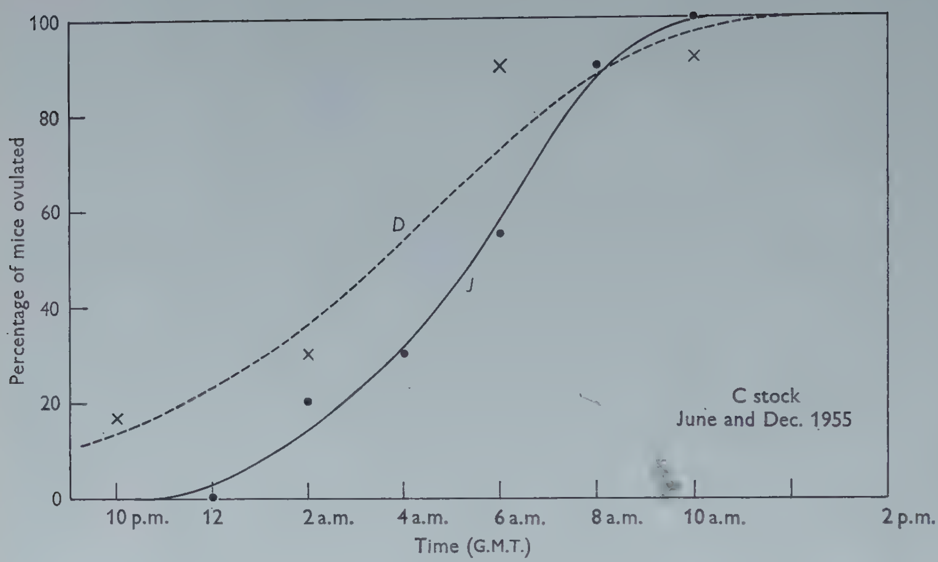

Fig. 3. A comparison of the periods required for ovulation in groups of C stock mice in June (curve J) and December (curve D). Natural diurnal light cycles.

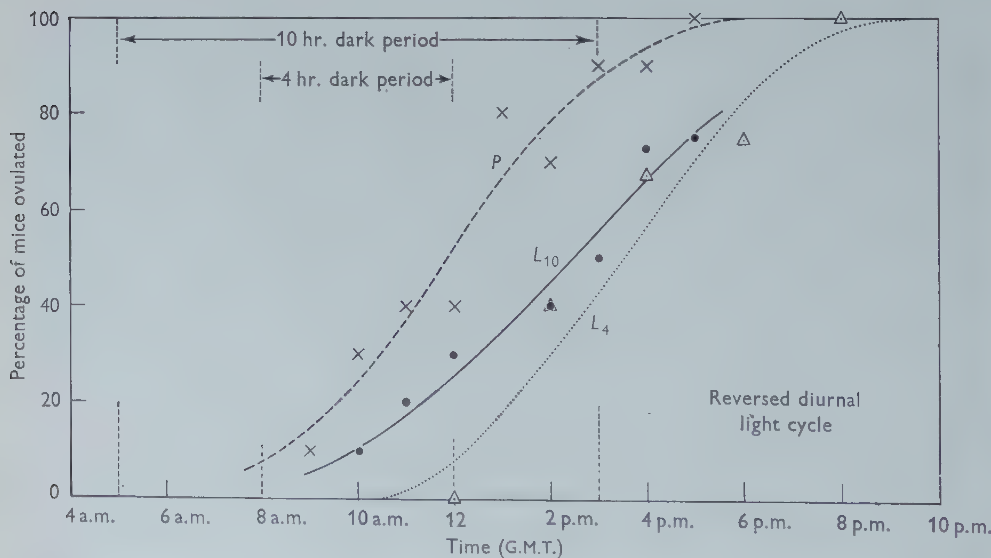

Fig. 4. Showing the progressive increase in the percentage of mice ovulated. Curve P illustrates the results from PCT stock mice, L_4 and L_{10} the results from L stock mice. P and L_{10}, mice kept under a reversed light cycle of 10 hr. dark/14 hr. light. L_4 mice kept under a 4 hr. dark/20 hr. light cycle.

ovulation occurred in this group of mice was thus about 9–10 hr. (curve P, Fig. 4). In C stock females, on the other hand, ovulation under the 10 hr. dark/14 hr. light régime appeared to occupy a much longer period. Five of the ten (C stock mice) killed at 9 a.m. had already ovulated and one of the ten killed at 5 p.m. had not (Table 4). Probit analysis of the results from the PCT females indicated that the mid-point of the ovulation period was 11.45 a.m. ± 10 min. and that the slope of the probit line was 0·378 ± 0·07.

Table 4. *The increase in the number of mice ovulated with time in PCT and C stock females maintained under a reversed diurnal light cycle of* 10 *hr. dark and* 14 *hr. light*

Time of day	PCT stock			C stock		
	No. of mice		Percentage of mice that had tubal eggs	No. of mice		Percentage of mice that had tubal eggs
	Examined	With tubal eggs		Examined	With tubal eggs	
9 a.m.	10	1	10	10	5	50·0
10 a.m.	10	3	30	12	5	41·7
11 a.m.	10	4	40	—	—	—
12 noon	10	4	40	12	7	58·3
1 p.m.	10	8	80	—	—	—
2 p.m.	10	7	70	10	5	50·0
3 p.m.	10	9	90	12	7	58·3
4 p.m.	10	9	90	10	9	90·0
5 p.m.	10	10	100	10	9	90·0

Ovulation began at about 8 a.m. in L stock females maintained under the 10 hr. dark/14 hr. light cycle. It was still in progress at the time of the last examination (5 p.m.). The results are set out in detail in Table 5 and are illustrated graphically in Fig. 4 (curve L_{10}). The slope of the probit line in this instance was 0·268 ± 0·063, and the mid-point of the ovulation period was estimated as 2·25 p.m. ± 15 min. The slope did not differ significantly ($\chi^2_1 = 1{\cdot}35$; $P < 0{\cdot}3$) from that found for ovulation in PCT stock mice, but there was a very significant difference (of 2 hr. 40 min.) between the mid-points of the ovulation periods, as may be seen from inspection of Fig. 4.

When L stock females were kept under the 4 hr. dark/20 hr. light régime—the mid-point of the dark phase being the same as in the 10 hr. dark/14 hr. light cycle—ovulation began at about 12 noon and reached completion at about 8 p.m. (Table 5). The slope of the probit line was found to be 0·42 ± 0·08, and the time by which 50% of the mice would have ovulated was 3.30 p.m. ± 18 min. This slope did not differ significantly ($\chi^2_1 = 2{\cdot}16$; $P < 0{\cdot}2$) from that derived for L strain mice kept under the 10 hr. dark/14 hr. light cycle. However, there was a significant difference between the mid-points of the ovulation periods of L strain mice under the two diurnal light cycles ($\chi^2_1 = 7{\cdot}22$; $P < 0{\cdot}01$).

In the animals kept under the natural diurnal light cycles the length of the dark phase was approximately 6½ hr. during the June experiment and 15½ hr. during the December experiment. These values have been calculated from the times of sunrise

and sunset in those months, except in the December experiment when artificial lighting was employed in the animal house until about 5.20 p.m. There are thus three sets of data in which to compare ovulation under a diurnal cycle involving a long dark phase (10–15½ hr.) and under a cycle with a short dark phase (4–6½ hr.).

Table 5. *The increase in the number of mice ovulated with time in females of L stock maintained under reversed diurnal light cycles*

Time of day	10 hr. dark/14 hr. light cycle				4 hr. dark/20 hr. light cycle			
	No. of mice			Percentage of mice that had tubal eggs	No. of mice			Percentage of mice that had tubal eggs
	Examined	With tubal eggs	With ⩾8 tubal eggs		Examined	With tubal eggs	With ⩾8 tubal eggs	
10 a.m.	10	1	1	10·0	—	—	—	—
11 a.m.	10	2	1	20·0	—	—	—	—
12 noon	10	3	3	30·0	15	0	0	0
2 p.m.	10	4	4	40·0	15	6	6	40·0
3 p.m.	10	5	4	50·0	—	—	—	—
4 p.m.	11	8	7	72·7	15	10	8	66·7
5 p.m.	20	15	12	75·0	—	—	—	—
6 p.m.	—	—	—	—	16	12	10	75·0
8 p.m.	—	—	—	—	10	10	10	100

These are the results from C stock (June and December 1955), L stock (June and December 1955), and L stock (4 hr./20 hr. cycle and 10 hr./14 hr. cycle). The rate of increase in the proportion of females that had ovulated in the populations studied is indicated by the slopes of the probit lines which were:

Short dark phase	Long dark phase	Stock
0·405 ± 0·075	0·235 ± 0·067	C
0·42 ± 0·07	0·286 ± 0·048	L
0·42 ± 0·08	0·268 ± 0·063	L

When these three sets of data were separately compared (see above) the differences between the slopes were not statistically significant. However, when the data were combined, the difference between the slopes obtained under cycles with a short dark phase and those obtained under cycles with a long dark phase was significant ($\chi^2_1 = 7{\cdot}29$; $P < 0{\cdot}01$). It may be concluded therefore, that ovulation occupies a longer period in an outbred mouse population when there is a relatively long dark phase in the diurnal light cycle, than in a similar population maintained under a cycle with a short dark phase.

The relationship between the mid-point of dark phase and the mid-point of the ovulation period (i.e. when 50% of the mice had ovulated) in the same three sets of data should also be summarized. In each case, the mid-point of the ovulation curve was later than the mid-point of the dark period; the intervals were:

Short dark phase	Long dark phase	Stock
4 hr. 45 min. ± 6 min.	1 hr. 55 min. ± 71 min.	C
4 hr. 0 min. ± 7 min.	4 hr. 10 min. ± 9 min.	L
5 hr. 30 min. ± 18 min.	4 hr. 25 min. ± 15 min.	L

The means for the two groups were 4 hr. 45 min. and 3 hr. 30 min. respectively.

The time required for ovulation in any one mouse. The mean number of eggs in forty-five L strain females killed after ovulation had been completed was 11·8 eggs per mouse. Only three of the mice had less than eight eggs. In seventy-five females from C strain, the mean number was 10·9 eggs per mouse, and six of the mice had less than seven eggs. These data were used to obtain an estimate of the average time required for ovulation *in any one mouse*. Curves similar to those shown in Figs. 2, 3 and 4 were drawn relating the percentage of mice with eight or more eggs (for L stock females) or with seven or more eggs (for C stock females) with time. Measurements were then made of the interval along the time axis between these curves and the corresponding curves (given in the text-figures) in which was plotted the percentage of mice with *one* or more eggs ovulated. The average intervals found for the six groups of L and C stock mice corresponded to 0·68, 0·32, 0·18, 0·40, 0·76 and 0·40 hr. The mean of these values is 0·46 hr. and this is taken to mean that the ovulation of the first seven or eight eggs (out of a total of 11–12 eggs) occupied about 0·5 hr. By a similar method Austin & Braden (1954) arrived at an estimate of 1 hr. for the ovulation of the first six eggs (out of a total of 9–10 eggs) in the rat.

DISCUSSION

It has been shown for a number of species of birds and mammals that the time of the year when the breeding season commences is determined largely by the seasonal changes in day length (for example, Rowan, 1927; Baker & Ranson, 1932; Bissonette, 1932; Yeates, 1949). In polyoestrous animals (such as the rat, mouse and hamster) evidence has been adduced to show that the period of the *day* during which the eggs are shed is also regulated by the time relations of the diurnal light cycle (Snell *et al.* 1940, 1944; Austin & Braden, 1954; Austin, 1956). The present results indicate that this period is affected by changes in the length of the day (i.e. the light period), as well as by reversal of the periods of light and darkness. With natural light cycles it is difficult to define the onset and end of the period of darkness for it is never abrupt and may be very gradual, especially at high latitudes where the summer twilight is long. Except in the December experiments, when artificial lighting was employed in the animal house until about 5·20 p.m., the times of sunset and sunrise have been taken as the onset and end, respectively, of the period of darkness. The mid-point of the dark period was estimated as 12.15 a.m. for the June experiment and 1.00 a.m. for the December experiments. The length of the dark period was approximately $6\frac{1}{2}$ hr. in June and $15\frac{1}{2}$ hr. in December. To adjust for the differences between the mid-points of the dark period in June and in December, 45 min. was added to the estimated 50% ovulation times in June experiments. When this was done the 50% ovulation times for L stock females examined in June and December no longer differed significantly, but the differences between the corresponding times for C stock females became significant ($P < 0{\cdot}025$). In L stock females kept under an artificial 4 hr. dark/20 hr. light cycle ovulation was significantly later than in similar females kept under a 10 hr. dark/14 hr. light cycle. The difference between the adjusted 50% ovulation times for C stock females was +2·85 hr., for L stock females under natural light cycles it was −0·12 hr., and

for L stock females under artificial light cycles it was $+1{\cdot}10$ hr. (using as the standards the 50% ovulation times for the cycles with a long dark phase). The results indicate that ovulation may be retarded by an hour or so when the dark phase of the diurnal light cycle is shortened by 6–9 hr., but that the mid-point of the ovulation period is probably more closely linked with the mid-point of the dark phase (or of the light phase) than with the onset or termination of the dark phase. The onset of the dark period was altered by 3 hr. in the artificial light cycle, and by about $4\frac{1}{2}$ hr. in the natural light cycle.

It seems safe to conclude on the evidence available, which has been recently reviewed by Harris (1955), that the effect of diurnal light cycles on the female reproductive system is mediated by the eyes and the central nervous system. Everett, Sawyer & Markee (1949) and Everett & Sawyer (1950) obtained results with normal, cyclic, rats that strongly suggest that neurogenic activation of the anterior pituitary takes place during a period of about 2 hr. on the day of pro-oestrus. This activation results in the release of ovulating hormone (presumably L.H.) from the anterior pituitary, and ovulation occurs 10–12 hr. later. Apparently the *day* on which pituitary activation occurs is determined by the relative rates of oestrogen and progesterone secretion by the ovary, for appropriate injections of oestrogen or progesterone resulted in ovulation a day earlier than normal in rats with a 5-day cycle (Everett, 1948). However, in such treated animals neurogenic activation of the pituitary took place at the same time of day as in untreated animals (Everett & Sawyer, 1949). It would seem that the centre responsible for neurogenic activation of the pituitary has a rhythmic diurnal fluctuation in sensitivity, and that this rhythm is largely controlled by the diurnal light rhythm. The sensitivity of the centre apparently only reaches the pitch necessary for pituitary activation—and, consequently, for ovulation—on the day when the circulating oestrogen and progesterone reach certain optimal levels. The present results indicate that this centre, or perhaps co-ordinating centres at higher levels, takes cognizance of both the beginning and the end of the dark (or light) phase.

In both of the comparisons made, ovulation occupied a longer period in winter than in summer, and this difference was reflected in the slopes of the probit lines. Similar results were obtained in mice maintained under artificially reversed diurnal light cycles. The increased time required for ovulation in mice kept under cycles with a relatively long dark phase was largely, if not entirely, the result of an increased variation between females in the time of the onset of ovulation, for the time required for ovulation of all of the eggs in any one female was relatively very short (less than 1 hr. probably), and there was no evidence to indicate that it was longer in winter than in summer. This implies that there was a variation between females in their reaction to a change in the diurnal light cycle. This variation appears to have a genetic basis, for definite evidence of variation between different stocks of mice in respect of the time of ovulation was found. Some of this variation was apparently related to differences in the ability of individual mice to adjust their internal neurogenic rhythm to the changed diurnal light cycle (as, for example, in C stock females placed under a reversed diurnal light cycle). However, much of the

variation between individual mice seems to have been due to varying relationships between the times of light and dark and the time of ovulation in females that were more or less completely adjusted to the diurnal light cycle. There would have been considerable variation in the genotype of the females within each stock, as well as between stocks for the mice were random-bred, and this is possibly one of the factors responsible for the excessive length of the ovulation period in the present stocks as compared with one studied previously (Braden & Austin, 1954). Also consistent with this hypothesis is the finding that under the artificial 10 hr. dark/14 hr. light cycle females of the PCT stock, which were rather more inbred than those of L and C stocks, not only ovulated earlier than females of L stock, but the period required for ovulation in the group also appeared to be less (see Fig. 4). Moreover, ovulation was found to occur over a more prolonged period in the mixed group of females studied in December 1954 than in the L and C stocks studied in December 1955. If the spread in the time of ovulation is to be imputed to genetic differences between mice, it would be expected that the period required for the completion of ovulation in a group of females from an inbred strain would be much shorter than in a group of outbred females. Unfortunately, data from inbred mice are not available, but in the rat ovulation in one inbred strain occupied only $1\frac{1}{2}$ hr. (Everett, 1948) as compared with about 4 hr. in a random-bred group of rats studied by Austin & Braden (1954). All these considerations support the hypothesis that in animals that display spontaneous ovulation the relationship between the environmental diurnal cycle of light and darkness and the diurnal rhythm of the neural centres involved in the ovulatory activation of the anterior pituitary, is determined by the genetic constitution of the animal.

SUMMARY

The period required for the completion of ovulation in groups of mated females has been studied in 615 mice from three random-bred stocks (L, C and PCT) and in 137 mice of mixed origin. The mice were examined after having been kept for some time under one of four different diurnal light cycles, viz. the natural diurnal light cycle in Edinburgh in December (1) and in June (2) and artificial, reversed, light cycles of 10 hr. darkness/14 hr. light (3) and 4 hr. darkness/20 hr. light (4).

The variation between mice in any one group was greatest when they were maintained under the cycles that had a relatively long dark phase: a period of 12–14 hr. was required for ovulation in groups of L and C stock mice under cycles with a dark phase of 10–15 hr. whereas groups of similar females maintained under cycles with a 4–6 hr. dark phase required only 8–9 hr. for ovulation. Probit analysis indicated that this difference was statistically significant.

In changing the length of the dark phase of the diurnal cycle the beginning of each phase was altered by $3-4\frac{1}{2}$ hr.; the mid-points of each phase were altered by less than 1 hr. The results suggest that the mid-point of the ovulation period was determined more by the mid-point of the dark phase than by its onset or completion.

Differences noted between stocks of mice in the mid-point of the ovulation period and in the ability to adjust quickly to an altered light cycle gave indication that the

neural mechanisms involved in the control of the time of ovulation in mice are modified according to the genetic constitution of the animal.

The mean interval between coitus and ovulation in females of L and C stocks under natural light cycles was found to be approximately 5 hr. The average time required for the ovulation of three-quarters of the total number of eggs shed in any one mouse (mean 11–12 eggs) was estimated as 0·5 hr.

These investigations were carried out during the tenure of a studentship granted by the Commonwealth Scientific and Industrial Research Organization, Australia. I wish to thank Prof. C. H. Waddington, F.R.S., for facilities and interest, and Dr R. A. Beatty for much helpful advice during the course of the work. Thanks are also due to Dr B. Woolf for help with the statistical treatment of the data, to Dr D. S. Falconer, Dr T. C. Carter and Dr N. Bateman for supplies of mice, and to Mr E. D. Roberts for drawing the figures.

REFERENCES

Austin, C. R. (1956). Ovulation, fertilization, and early cleavage in the hamster (*Mesocricetus auratus*). *J. R. Micr. Soc.* **75**, 141–54.

Austin, C. R. & Braden, A. W. H. (1954). Time relations and their significance in the ovulation and penetration of eggs in rats and rabbits. *Aust. J. Biol. Sci.* **7**, 179–94.

Baker, J. R. & Ranson, R. M. (1932). Factors affecting the breeding of the field mouse (*Microtus agrestis*). I. Light. *Proc. Roy. Soc.* B, **110**, 313–22.

Bissonette, T. H. (1932). Modification of mammalian sexual cycles: reactions of ferrets (*Putorius vulgaris*) of both sexes to electric light added after dark in November and December. *Proc. Roy. Soc.* B, **110**, 323–36.

Bissonette, T. H. (1938). The influence of light upon pituitary activity. *Res. Publ. Ass. Nerv. Ment. Dis.* **17**, 361–76 (quoted by Harris, 1955).

Braden, A. W. H. & Austin, C. R. (1954). Fertilization of the mouse egg and the effect of delayed coitus and of hot-shock treatment. *Aust. J. Biol. Sci.* **7**, 552–65.

Browman, L. G. (1937). Light in its relation to activity and oestrus rhythms in the albino rat. *J. Exp. Zool.* **75**, 375–88.

Clarke, W. E. Le Gros, McKeown, T. & Zuckerman, S. (1939). Visual pathways concerned in gonadal stimulation in ferrets. *Proc. Roy. Soc.* B, **126**, 449–68.

Everett, J. W. (1948). Progesterone and oestrogen in the experimental control of ovulation time and other features of the oestrous cycle in the rat. *Endocrinology*, **43**, 389–405.

Everett, J. W. & Sawyer, C. H. (1949). A neural timing factor in the mechanism by which progesterone advances ovulation in the cyclic rat. *Endocrinology*, **45**, 581–95.

Everett, J. W. & Sawyer, C. H. (1950). A 24-hour periodicity in the 'LH-release apparatus' of female rats, disclosed by barbiturate sedation. *Endocrinology*, **47**, 198–218.

Everett, J. W., Sawyer, C. H. & Markee, J. E. (1949). A neurogenic timing factor in control of the ovulatory discharge of luteinizing hormone in the cyclic rat. *Endocrinology*, **44**, 234–50.

Harris, G. W. (1955). *Neural Control of the Pituitary Gland*. London: Edward Arnold Ltd.

Hemingsen, A. N. & Krarup, N. B. (1937). Rhythmic diurnal variations in the oestrous phenomena of the rat and their susceptibility to light and dark. *K. danske Vidensk. Selsk. Biol. Medd.* **13**, (7), 5–61.

Hill, M. & Parkes, A. S. (1933). Studies on the hypophysectomized ferret. V. Effect of hypophysectomy on the response of the female ferret to additional illumination during anoestrus. *Proc. Roy. Soc.* B, **113**, 537–40.

Rowan, W. (1927). Migration and reproductive rhythm in birds. *Nature, Lond.*, **119**, 351–2.

Snell, G. D., Fekete, E., Hummel, K. P. & Law, L. W. (1940). Relation of mating, ovulation, and the oestrous smear in the house mouse to the time of day. *Anat. Rec.* **76**, 39–54.

Snell, G. D., Hummel, K. P. & Abelmann, W. H. (1944). A technique for the artificial insemination of the mouse. *Anat. Rec.* **90**, 243–53.

Yeates, N. T. M. (1949). The breeding season of the sheep with particular reference to its modification by artificial means using light. *J. Agric. Sci.* **39**, 1–43.

TEMPERATURE RELATIONS OF TENERAL DEVELOPMENT AND BEHAVIOUR IN *APHIS FABAE* SCOP.

By L. R. TAYLOR

Department of Entomology, Rothamsted Experimental Station, Harpenden, Herts

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INTRODUCTION

The period of relative quiescence which follows each ecdysis in arthropods varies greatly in duration in different stadia, in different individuals and in different species. It is associated, amongst other things, with hardening of the exoskeleton and also, at eclosion of the adult in alate insects, particularly with expanding and hardening of the wings; this must be completed before flight can take place. Thus the interval between eclosion and flight involves developmental processes which may be expected to respond to temperature in the way typical of other stages in insect development.

The word 'teneral' has been used to describe alate insects at about this time, although its usage varies between different orders. It has already been applied to the aphids with which this paper is mainly concerned, and it is convenient to adopt the name here and discuss its several meanings later. When teneral development is complete, flight may still be prevented, or delayed, by behaviour in response to physical factors such as temperature and light, and to biological factors such as the presence of adjacent insects. 'Teneral' is therefore used here to denote that stage between final ecdysis and the time when the alate aphid is capable of flight, external factors permitting.

Inhibition of flight, e.g. by low temperature, is an individual response; an insect will either take flight or not take flight at a given temperature on a particular occasion. Seen in a natural population this has the form of a frequency distribution against temperature. For example, in *Aphis fabae* the response curve rises from zero at 15·5° C. to a maximum at 17·3° C. and falls again to zero at 20° C. (see C. G. Johnson & Taylor, 1957).

As a consequence any attempt to measure the length of the teneral period at *constant* temperatures in the laboratory, without a previous knowledge of the response curves for behaviour inhibition and development, would probably lead to erroneous results below 20° C. At these temperatures flight-mature but flight-inhibited insects would not be distinguishable from flight-immature insects, and the resultant curve for time against temperature would go off to infinity at about 15·5° C.—the development velocity being apparently zero when there is no take-off. The concept of separate functions for behaviour and for the teneral period as a development stage would not emerge.

The independent effects of temperature on development and on behaviour were, in fact, found and segregated by analysis of the data obtained in field experiments during which the temperature was constantly changing over a wide range (*c.* 7–34° C.), along with other climatic factors. By this analysis it is possible to show that the variation in teneral time in *A. fabae* in a bean crop can be largely accounted for by four simple assumptions.

(1) The rate of development during the teneral period is logistically related to temperature between 10 and 30° C. irrespective of whether the temperature is constant or varies about a mean value.

(2) Inherent individual variations are imposed upon this trend.

(3) Take-off is inhibited at temperatures below *c.* 16° C. as measured at 1 ft. in the crop.

(4) Take-off is inhibited at light intensities below *c.* 100 ft.-candles.

This analysis alone does not, of course, prove that these are the direct causative factors, but once the development and behaviour factors have been segregated it is possible to confirm the results at constant temperatures in the laboratory.

The logistic formulation of the rate of development has been tested in the laboratory under conditions of constant and variable temperature and found to describe adequately the temperature relation up to the optimum response at about 28° C. Above this value neither were the field data sufficient to give accurate results, nor is the type of curve fitted appropriate to data showing a falling rate. Nevertheless, the range of temperatures, over which the constant-temperature treatment and the highly variable field treatment yielded almost identical developmental rates, is adequate evidence that temperature variations *per se*, and other climatic factors correlated with temperature (at least of the order found in nature), have little or no effect on developmental rates.

There are consequently four main issues in the present paper. First, that it is possible to distinguish between the effects of development and of behaviour in quantitative studies on populations in the field as well as in investigations of the behaviour of individuals. Secondly, that the teneral period, between eclosion and flight, can be treated largely as a developmental stage. Thirdly, as mentioned above, that this stage is controlled in its duration mainly by a quantity of heat, and that other climatic factors, especially rate of change of temperature, have negligible effect. Fourthly, that such temperature relationships can be analysed from field data obtained in highly variable climatic conditions with an accuracy closely approaching that obtained in the laboratory, without the attendant restrictions imposed by arbitrary control of environment and the consequent difficulty of application to natural conditions. Considerable attention has therefore been given to the method of analysis.

GENERAL METHOD

The material has been extracted from the tabulated raw data in C. G. Johnson, Taylor & Haine (1957), wherein the experimental site and procedure are described in detail, and where a full temperature table in half-hour means for the whole

experimental period is given. Briefly, alienicolae of *A. fabae* were inspected at frequent intervals *in situ* on the leaves of a naturally infested field-bean crop in a walled garden, and were marked with paint in batches as they moulted. The time of departure of these alatae (flight) was also recorded, again in batches between two successive inspections. All the insects marked in one batch did not necessarily depart together, and hence the history of an individual cannot be traced. Each experiment consisted of marking several successive batches with the same colour over a period of about 4 hr. (a 'colour group'), and it is the mean time of such a colour group from moulting to flight that is considered. Variation in individual behaviour is an additional factor eliminated by this treatment, but it has been studied independently in laboratory experiments (p. 203).

The data for each experiment were thus reduced to a single record, the mean teneral period, from moulting to flight, which was plotted against the arithmetic mean temperature for the interval. This gave a hollow curve with wide scatter, but sufficient to show that temperature was a major factor in determining the teneral period. From this stage, by a succession of approximations described later, the reciprocal curve (i.e. the rate of development curve) was gradually arrived at: batches which were estimated to have completed maturation in darkness or low temperature, and consequently to have been delayed by behaviour inhibition, were eliminated; until, finally, almost the whole variation in teneral time was accounted for. Inhibiting light and temperature levels were obtained, independently of these data, from trap catches in the same crop.

CLIMATIC FACTORS INHIBITING TAKE-OFF

Take-off is inhibited during the night as can clearly be seen from suction trap catches (C. G. Johnson, 1952). This spuriously lengthens the teneral period of aphids maturing during the night, and such insects cannot be used in the formulation of the temperature-velocity curve for teneral development. It is therefore necessary to determine the climatic limits beyond which take-off inhibition occurs. Such limits will of necessity be arbitrary, depending upon the means available for their measurement.

Temperature

The known temperature relations of aphid flight activity recently reviewed by Moericke (1955) are of little use for this purpose, because the threshold chosen must be a single temperature level measured at a specific place in relation to the aphids.

A suction trap (Taylor, 1951) was operated in the centre of the experimental crop with the collecting inlet just below the crop level. Such a trap may be used to give a direct density estimate (C. G. Johnson & Taylor, 1955) of flying insects per 9000 cu.ft. ($\pm 5\%$) of air per half hour (Taylor, 1955). Trap catches consist of insects just leaving the crop on their initial flight (see later).

Air temperature records for 1 and 4 ft. levels in the crop were also available, and from these the maximum temperature reached in any half-hour could be read.

The aphid density, plotted against the maximum temperature for half-hour periods in the mornings and covering a range of 12–20° C., is shown in Fig. 1 (4 ft.) and Fig. 2 (1 ft.). The diagrams are interpreted thus; as the sun rises and the tops of the plants are warmed, the aphids in the crown of the plants take flight sporadically, as temperature permits. This temperature is recorded by the thermometer at 4 ft., and the threshold is the temperature at which the first few aphids are caught, i.e. at 15–17° C. But full-scale catches cannot be expected until the whole crop is raised above the threshold temperature as recorded by the thermometer at 1 ft. Consequently, in Fig. 2, the temperature at which large catches (20–30) begin may be taken as the threshold. Considered together these two diagrams give an operational threshold of 16° C. at 1 ft.; the four largest catches below 16° C. fall on the same day, an occasion when the thermometer at 1 ft. registered 15–16° C. for several hours and some parts of the crop must have had temperatures above 16° C.

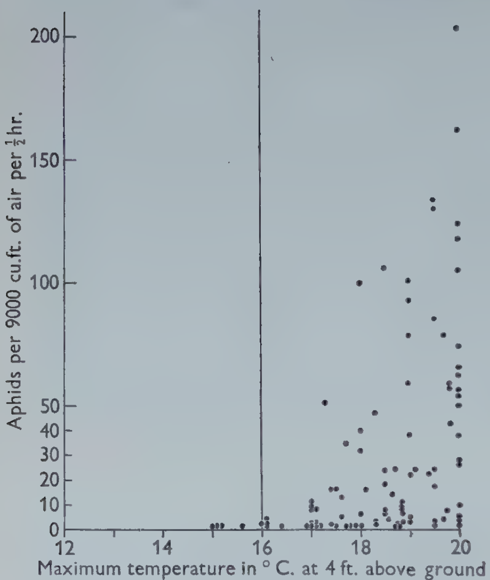

Fig. 1

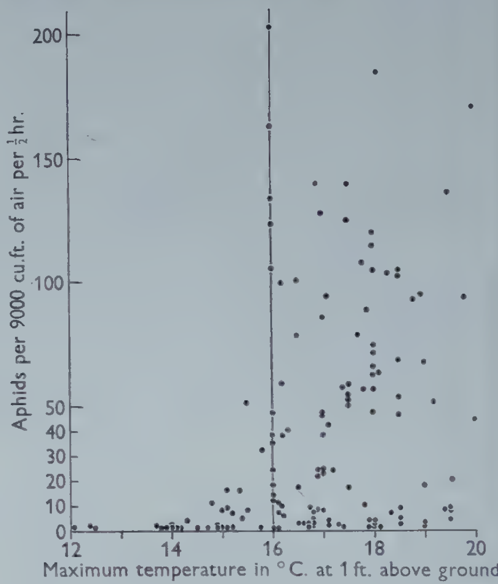

Fig. 2

Figs. 1, 2. Each point represents the mean aerial density for ½ hr. plotted against the maximum temperature during that ½ hr. Samples taken each morning for 20 days.

This is not the mean of the take-off threshold temperature distribution as measured in the laboratory (C. G. Johnson & Taylor, 1957); that gives the actual temperatures to which the aphids respond. The temperature threshold for take-off used here is 16° C. at 1 ft. in a bean crop; no appreciable take-off occurs below this temperature.

Light

Laboratory experiments by Broadbent (1949) indicated that flight is completely inhibited in *Myzus persicae* Sulz. at just below 1 f.c. and in *Brevicoryne brassicae* L. at about 1 f.c. (fluorescent lamp); full activity is approached at intensities above

100 f.c. There was no information about *Aphis fabae*. A method similar to that employed for temperature was therefore used to find the light threshold. The aphid density was plotted against the time (15.00–24.00 hr., G.M.T.), at the end of the day when light was failing but temperature still high (Fig. 3); no records at temperatures below 16° C. were used. Only in a general way can light be associated with time; dull days will reach the same light value earlier in the day. The fall-off in catch at 19.00–19.30 hr. G.M.T. is, however, fairly clear, although it must occur earlier than this on some occasions. The experimental crop was surrounded on the south and west by trees and the light intensity would be slightly lower than standard, i.e. 10–20 f.c. at sunset (*c.* 20.10 hr.) instead of 33 f.c. and about normal, i.e. 0·4 f.c. at the end of civil twilight (*c.* 21.00 hr.).

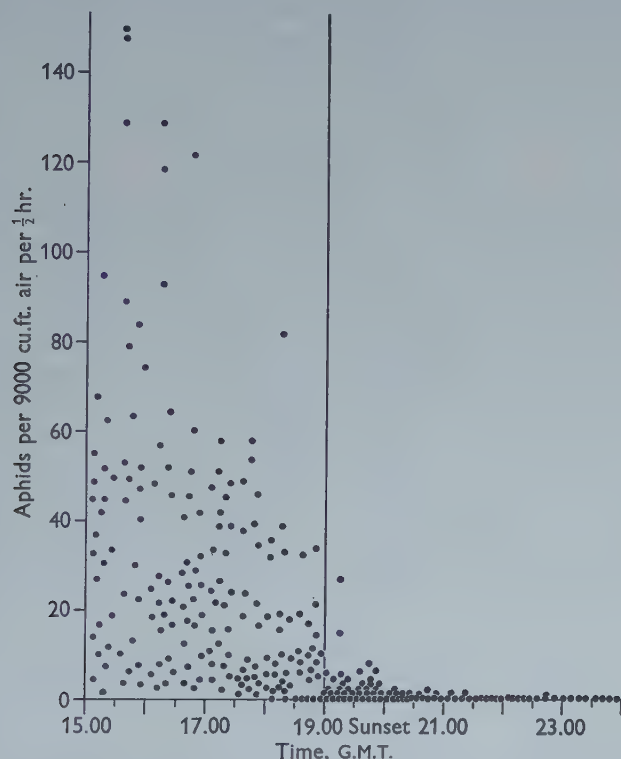

Fig. 3. Each point represents the mean aerial density for ½ hr. at the mean time shown. Samples taken each evening for 20 days, at temperatures above 16° C. Sunset indicated at a mean position.

Thus the values are of the same order as for Broadbent's *Brevicoryne brassicae* and *Myzus persicae*: a reduction in activity commencing about 3½ hr. before sunset (*c.* 1000 f.c.) and falling with increasing steepness to reach negligible proportions at about 1 hr. before sunset (*c.* 10 f.c.), with complete cessation by the end of civil twilight (0·4 f.c.). The light threshold used is 1 hr. before sunset.

There may be a slight excess in the numbers of aphids recorded in the last few catches of the day owing to the escape of insects from one disk of the trap to the next (C. G. Johnson & Taylor, unpublished), but the effect must be very small, as can be seen by inspection of the catches in C. G. Johnson *et al.* (1957).

Wind and humidity

Wind speed is a significant factor in delaying take-off but, unless combined with low temperature, probably rarely prevents it (Haine, 1956). In these experiments the wind rarely exceeded 4 m.p.h. and was usually below 2 m.p.h.

Humidity is probably not a factor in take-off behaviour, although sudden changes may have a temporary effect on activity (Broadbent, 1949). There are apparently no such sudden changes in the humidity in the present record, as measured by a whirling hygrometer between the plants, and this must reflect the condition at the leaf surface to a large degree.

EXPERIMENTAL ANALYSIS

Extraction of the data

The raw data are given in Table 2 of C. G. Johnson *et al.* (1957), and Tables 1 and 3 of this paper show the data after condensation. The method of construction of these tables must be described, for it involves an arbitrary step in the analysis.

In the original table the time of marking of each experimental batch of colour-marked aphids is given. Moulting, which occurred during the interval between marking and the previous inspection, is assumed to have taken place midway between these two inspections. The mean moulting time (Table 1, col. 4) for the whole experiment is a weighted mean of all such batches.

Departure times (Table 1, col. 7) are treated in the same way when the rate of departure plotted against time gives a fairly normal curve, as it generally does. When the departures are split into two groups by nightfall however, departure curves are usually very skew. All experiments were therefore drawn as histograms, against time, and when these were skew an attempt was made to find the mode. Assessment of the mode was then, of necessity, arbitrary and was found from a curve fitted by eye; the experiments in which this was done are indicated in the table. The possible errors introduced are not large, but obviously cannot be assessed. For this reason and also because the history only of batches not of individual aphids can be followed, statistical analysis of the means is not possible. Exps. 45 and 46 were added together to facilitate the assessment of means, for this is more critical when the teneral time is short as it was here.

When the departures extended over 2 days and the two departure groups were of equivalent size it was assumed that, in general, the first to moult were the first to depart, and the experiment is accordingly divided in two (see Johnson *et al.* 1957). This leads to a slight bias in rate of development between the two groups owing to the non-randomness of the segregation, the first group maturing rather more rapidly than normal, and the second group more slowly. Where either of these two groups

was too small to be used alone it was included in the gross mean, but the inhibiting hours of darkness and cold were eliminated to maintain a balanced distribution.

Table 1. *Condensed data for field experiments used in estimating teneral time*

xp. no.	Moulting times, G.M.T.			Flighting times, G.M.T.			No. aphids	Teneral time	Thermal summation	
	Distribution limits		Mean	Distribution limits		Mean			Arith-metic	Logistic
	From	To		From	To					
	12.00	18.00	15.7/2	05.00	08.00	11.3/4	9	43·6	145	90·9
	13.30	19.30	17.4/5	06.15	15.00	10.3/6	23	16·9	118	95·0
	04.15	10.15	06.9/6	13.00	19.00	15.9/6	40	9·0	139	101·2
	04.15	10.15	06.6/8	12.30	11.15	18.7/8	34	12·1	106	96·0
*	05.15	07.15	06.3/9	13.30	06.15	16.5/9	39	10·2	112	98·7
*	06.15	08.15	07.3/9	11.00	19.00	17.0/9	42	9·7	107	99·1
	11.30	19.30	14.4/9	06.30	15.30	09.6/10	44	19.2	130	104·8
	15.30	19.30	16.9/9	07.00	14.30	11.7/10	32	18·8	120	99·0
	05.15	08.15	06.7/10	13.30	18.30	16.2/10	42	9·5	123	101·4
*	06.15	09.00	07.7/10	11.30	18.30	16.5/10	59	8·8	123	102·3
	10.15	17.30	14.5/10	06.15	17.30	11.0/11	55	20·5	151	112·7
	09.45	13.00	11.4/11	09.30	15.00	12.2/12	29	24·8	132	106·0
	05.15	09.45	06.7/12	18.00	16.45	11.3/13	70	28·6	152	103·2
	09.45	16.00	13.3/12	18.00	19.15	14·2/13	42	24·9	144	102·9
*	13.30	18.30	16·0/12	06.15	18.30	14.8/13	29	22·8	138	96·2
*	13.30	18.30	16.0/12	08.00	16.45	14.4/13	22	22·4	138	90·8
	09.45	16.30	12.5/13	07.30	15.30	11.2/14	39	22·7	138	102·6
	09.45	13.00	12.3/14	09.00	18.30	14.4/15	30	26.1	107	99·8
	05.15	12.30	07.8/15	08.00	16.15	10.9/16	49	27·1	119	105·7
	07.15	11·00	09.1/15	19.00	16.15	10.0/16	51	24·9	105	94·3
	09.15	16.30	12.7/15	16.30	19.15	13.1/16	61	24·4	101	94·7
	07.15	11.00	09.1/16	17.40	11.45	09.6/17	20	24·5	135	104·3
	09.15	13.30	11·8/16	18.30	18.30	12.6/17	33	24·8	130	104·0
	09.15	16.30	11·9/17	07.00	16.45	12.1/18	26	24·2	145	103·0
	16.30	18.00	17.3/17	12.30	07.30	15.8/18	25	22·5	145	96·8
	10.15	18.30	13.9/18	19.00	16.45	10.7/19	37	20·8	134	105·9
*	14.30	18.00	16.3/18	07.30	19.15	12.4/19	32	20·1	142	107·4
	15.00	19.00	17.2/18	10.00	19.15	12.2/19	29	19·0	130	97·9
	12.30	19.00	14.8/19	06.30	15.00	08.8/20	45	18·0	125	107·4
	13.30	18.00	15.7/19	06.30	19.15	09.3/20	33	17·6	123	96·4
+46	06.15	11.00	08.5/20	11.00	09.00	16.8/20	57	8·3	137	101·5
	15.30	18.00	16.6/20	18.00	18.00	08.5/21	18	15·9	133	105·9

Mode used; not mean.
n columns 4 and 7 the mean times are given as: time (G.M.T.) to the first decimal of an hour/date (July 1952).

Small isolated departures, dissociated from the main mass, are not included in the assessment of means, as it is believed that those departing soon after marking were disturbed either by the marking or by movement of other aphids, and those remaining very late may have been damaged. In Table 1 this is indicated by the limits of time for the mean (cols. 2 and 3, and cols. 5 and 6), which exclude such odd aphids, and by the slight reduction in numbers (col. 8) as compared with this value in the original table. The total number of aphids is reduced from 1248 to 1196.

Table 1 does not include those experiments estimated to be delayed by behaviour pattern. These are presented in Table 3 (see p. 201), but it should be emphasized that at each stage in the analysis these experiments were considered individually before they were discarded.

Method of curve-fitting

During the analysis the process of curve-fitting, which takes place in several stages, runs parallel with that of segregating behaviour from development; they are in fact complementary. It is simpler, however, to describe curve-fitting first, dealing only with those experiments in which flight was not evidently delayed by low temperature or light intensity.

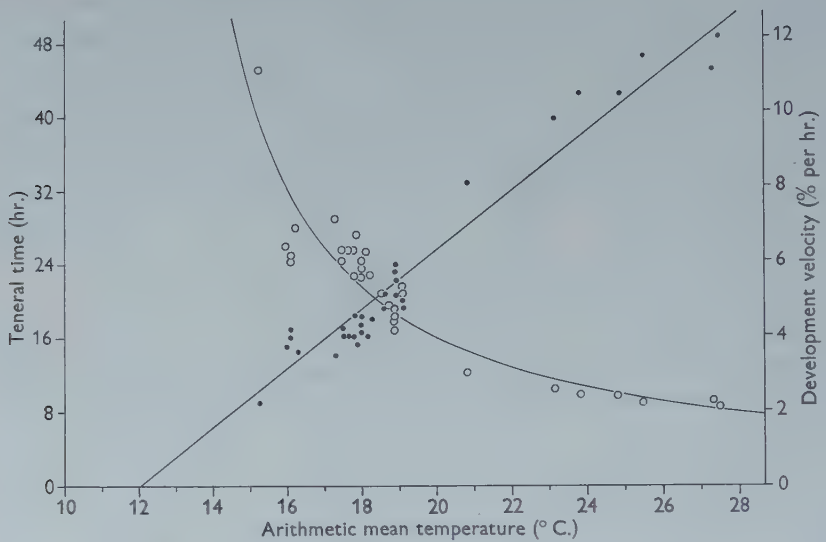

Fig. 4. Teneral time and development rate in nature using arithmetic means of variable temperatures.

Fig. 4 shows the condensed data for teneral period given in Table 1, col. 9, plotted against the *arithmetic* mean temperature during the teneral period obtained from the table in C. G. Johnson *et al.* (1957). The reciprocals of teneral period, or development velocities, for each experiment are also plotted and to these has been fitted the straight regression line:

$$v = 0{\cdot}78\ (\theta - 12{\cdot}0), \tag{1}$$

where v = development velocity (% per hour) and θ = empirical temperature (arithmetic mean) in ° C. This gives a fitted curve (rectangular hyperbola) for the teneral period:

$$t\ (\theta - 12{\cdot}0) = 128, \tag{2}$$

where t = teneral period in hours. The so-called thermal constant, 128 degree-hours, implies that, from the time of eclosion, the sum of hourly mean temperatures in excess of 12° C. (thermal summations) reaches 128 upon completion of teneral development. The observed thermal summations for each separate experiment is given (Table 1, col. 10), and the estimated thermal constant is the mean of these summations. The thermal sums vary from 100 to 150, and an expected time for maturation in variable temperatures assessed from these equations would be liable to a correspondingly large error.

This is because the relationship of v to θ is not adequately accounted for by the straight-line regression; it is in fact better described by a sigmoid curve, but such a curve cannot be fitted to the points as they stand. Each point represents the mean period of time for a group of insects, plotted against the arithmetic mean temperature during that period, implying that development during that time is linearly related to temperature. The temperature may in fact vary over almost the whole range under consideration during the time expressed by a single point, and if the fitted line is curvilinear, then the individual arithmetic means become invalid, and the points move. In other words, a curve must first be estimated and any means taken must be allowed due weight for the development rate appropriate to each temperature along that curve, i.e. the developmental increment equivalent to each temperature, the temperature equivalent, must be used in assessing means, instead of the measured temperature.

The method of obtaining means by use of equivalent, or effective, temperatures may be clarified by a more straightforward example. If a process is linearly related to temperature, e.g. the observed values at 10:20:30° C. are 2:4:6 per hour; then $\frac{1}{2}$ hr. at 10° + $\frac{1}{2}$ hr. at 30° gives an observed value of $(1+3)=4$ for 1 hr. This is equivalent to 1 hr. at $(10+30)/2=20$° C., i.e. the arithmetic mean temperature gives the correct temperature for the observed value.

If the process is logarithmically related to temperature, e.g. the observed values at 10:20:30° C. are 2:8:11·5 per hour, then $\frac{1}{2}$ hr. at 10° + $\frac{1}{2}$ hr. at 30° C. gives an observed value of $(1+5{\cdot}75)=6{\cdot}75$ for 1 hr. This is *not* the value observed at 20° (the arithmetic mean temperature) but at the log mean (or geometric mean) temperature, i.e. at antilog $\{\frac{1}{2}(\log 10+\log 30)\}=17{\cdot}4$° C. In other words, the true mean temperature for a logarithmic relation is found by converting the actual temperature to temperature equivalents using logarithmic tables, taking a mean, and converting back.

Exactly the same process is necessary to find the true mean for a logistic relationship, except that the logistic tables are not published but must be made from the curve used. Even if the curve used is not exactly logistic, or is not fitted accurately, the results obtained will approach more closely to the truth than will arithmetic means. *The mere fact that the correct functional relationship is not known beforehand is no justification for using arithmetic means except as a purely provisional estimate.*

The errors introduced by using arithmetic means have been analysed, in an investigation similar to this, by Andrewartha (1944). The curve from which he obtained his temperature equivalents was based on laboratory data. In the present analysis a process of successive approximation was adopted.

As a first approximation an arbitrary sigmoid curve was drawn through those arithmetic means in Fig. 4 with the smallest temperature range, i.e. in which distortion was minimal. Using this curve a table of temperature equivalents was made by reading off the appropriate development rate, or temperature equivalent, at each temperature. The observed $\frac{1}{2}$-hourly temperatures were then converted by this table to temperature equivalents, new means were taken and converted back to actual temperatures by the same table, and these new points plotted. To this

second estimate was then fitted a formulated (logistic) curve, and from this another table of logistic temperature equivalents obtained. Logistic means were taken and the process repeated successively, each time using a new logistic conversion table to obtain means from the original, observed, temperature record. By noting the movements of the points in relation to the line, a curve was eventually found which gave means equally distributed on each side of the line, and of minimum deviation. The final rate of development, or equivalent temperature conversion table for these aphids, is given in Table 2.

Table 2. *Conversion table for degrees Centigrade to equivalent semi-logistic developmental units for* Aphis fabae

Degrees Centigrade	0·0	0·1	0·2	0·3	0·4	0·5	0·6	0·7	0·8	0·9
	Semi-logistic equivalent									
9	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1
10	0·1	0·1	0·1	0·1	0·1	0·1	0·2	0·2	0·2	0·2
11	0·2	0·2	0·2	0·2	0·3	0·3	0·3	0·3	0·3	0·3
12	0·3	0·4	0·4	0·4	0·4	0·4	0·4	0·5	0·5	0·5
13	0·5	0·5	0·5	0·6	0·6	0·6	0·6	0·7	0·7	0·7
14	0·7	0·8	0·8	0·8	0·9	0·9	0·9	1·0	1·0	1·1
15	1·1	1·2	1·2	1·3	1·3	1·4	1·5	1·5	1·6	1·7
16	1·8	1·9	2·0	2·0	2·1	2·2	2·3	2·4	2·5	2·6
17	2·7	2·8	3·0	3·1	3·2	3·3	3·4	3·5	3·7	3·7
18	3·9	4·0	4·2	4·4	4·5	4·6	4·8	5·0	5·1	5·2
19	5·4	5·5	5·7	5·8	6·0	6·1	6·3	6·5	6·6	6·8
20	6·9	7·1	7·2	7·4	7·5	7·7	7·8	7·9	8·1	8·2
21	8·4	8·5	8·6	8·8	8·9	9·0	9·1	9·2	9·3	9·5
22	9·6	9·7	9·8	9·9	10·0	10·0	10·1	10·2	10·3	10·4
23	10·5	10·5	10·6	10·7	10·8	10·8	10·9	10·9	11·0	11·0
24	11·1	11·2	11·2	11·3	11·3	11·3	11·4	11·4	11·4	11·5
25	11·5	11·5	11·6	11·6	11·6	11·7	11·7	11·7	11·7	11·8
26	11·8	11·8	11·8	11·8	11·9	11·9	11·9	11·9	11·9	11·9
27	11·9	11·9	12·0	12·0	12·0	12·0	12·0	12·0	12·0	12·0
28	12·0	12·0	12·0	12·0	12·0	12·0	12·0	12·0	12·0	11·9
29	11·9	11·9	11·9	11·9	11·8	11·8	11·8	11·7	11·7	11·7
30	11·6	11·6	11·6	11·5	11·5	11·5	11·4	11·3	11·2	11·1
31	11·0	10·9	10·8	10·6	10·5	10·4	10·2	10·1	9·9	9·8
32	9·6	9·5	9·3	9·1	8·9	8·7	8·5	8·3	8·0	7·7
33	7·4	7·1	6·8	6·4	6·0	5·5	4·9	4·2	3·4	2·1
34	0	0	0	0	0	0	0	0	0	0

From 9 to 28° C. the equivalents are derived from the fitted logistic curve. Above 28° C. the equivalents are from the semi-logistic curve in Fig. 6.

Thermal sums from successive tables were used as indicators for the improvement of the curve used, and the final values are given in col. 11, Table 1. They may be compared with the arithmetic thermal sums (col. 10). Both have been halved to make the theoretical thermal constant 100 for col. 11, this being necessitated by the use of $\frac{1}{2}$ hr. periods; consequently col. 11 is also expressed directly as a percentage of the expected constant. As the accuracy of the fitted curve is judged by the closeness with which the thermal sums approach a mean (the thermal constant), the value

of this increased accuracy is perhaps best expressed by the standard deviations which are:

	Thermal constant	
	Mean	S.D.
Straight line (arithmetic means)	128·9	14·0
Sigmoid curve (logistic means)	100·9	5·1

This means that although expression (2) may be useful for comparison of the temperature relations of the teneral stage in different aphid species, or in different phases of the same species, it is of little value in forecasting the time of flight (± 22 % for the 95 % confidence limits); the second curve makes this possible, within reasonable ecological limits (± 10%) (see C. G. Johnson *et al.* 1957).

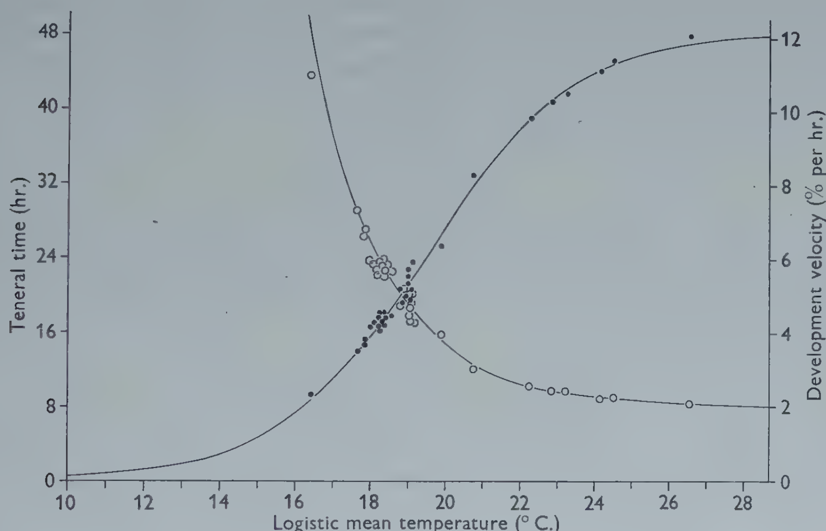

Fig. 5. Teneral time and development rate in nature fitted to logistic temperature-velocity curve.

The marked aphids were nearly all at the tops of plants on the edge of the crop and would therefore respond to air temperatures recorded at about 4 ft. But since the mass of aphids in the crop, and hence the trap catch, responded more clearly to the 1 ft. air temperature, it is this record which is reproduced (Table 1, Johnson *et al.* 1957). The present curve has been fitted to both records with identical results except that the absolute level differs by 2° C. Therefore the 1 ft. record was finally used, since it is published, and the resultant curve raised 2° C. to bring it to the 4 ft. level, to which these aphids were more nearly responding. Consequently the sums for col. 10, Table 1, should be considered in excess of 10° C. when dealing with the published temperature record.

The curve used

The development-temperature relationship follows a sigmoid curve from the cold death-point to an optimum and then falls quickly as first shown by Verworn (1894). This curve is very like the Pearson type 1 curve of negative skew distribution

(Elderton, 1953), and it seems reasonable to attribute the resultant temperature response to the statistical summation of individual responses of innumerable biochemical processes, each point on the curve being an index of the efficiency with which the processes contributing to speed of development operate at that temperature. The curve could therefore be considered as a distribution curve of relative efficiency with an optimum at the point at which the greatest number of processes are working with, and co-ordinated with, the greatest efficiency. The Pearson type 1 curve is, however, difficult to fit to such irregular data.

Many of the curves produced in the past to relate these two variables have been linear transformations, some based on hypothetical functional relationships, but the best description of the rising part of the curve is given by Davidson's (1942) logistic, which is purely empirical and closely approximates to the rising part of the negative skew distribution curve. It covers most of the range required here and is easy to fit; this is the curve used, fitted by the 3-point method of Davidson (1944).

The logistic expression used to produce Fig. 5 is

$$\frac{100}{t}=\frac{12\cdot 25}{1+e^{1\cdot 934-0\cdot 5066\theta}}. \tag{3}$$

The optimum occurs at *c.* 28° C. (see later), but there are so few experiments extending above 30° C. that any deviation above this would not affect the result. Similarly, below 10° C. the curve has almost reached zero but the records are too few to define a cut-off.

The segregation of development and behaviour

The time between moulting and flight may be influenced in two ways: either by factors affecting the rate of teneral development, or by factors affecting behaviour at the end of the teneral period.

If there are climatic factors other than temperature involved in *development* they should appear, not as additions to teneral time associated with the level of the factor at the time of flight, but as logarithmic deviations associated with the mean factor over the whole teneral period. No such relationship has been found with wind speed, or humidity or rate of change of temperature. It is therefore assumed that whatever other climatic factors control development during the teneral time, they are so closely associated with temperature that they appear in the logistic temperature regression.

Behaviour, on the other hand, operates as an inhibitor to flight, *adding* to the teneral time. This addition will not be dependent upon the mean level of the activating factor during the measured time; it will be evident as a release of activity at some *particular level* of the factor concerned at the moment of departure. Conversely if flight appears to take place at a particular temperature level, or level of light intensity, the teneral time apparently having continued longer than expected, and the expected termination of flight having occurred at an inhibiting level of the factor concerned, then behaviour could be postulated as the cause.

Behaviour is already taken into account to some extent in removing those experiments in which the teneral time theoretically terminated at temperatures below 16° C., or after 19.00 hr. G.M.T., this being done anew after each successive estimate of teneral time. Residual temperature effects above 16° C., effects due to wind speeds, and effects of rate of change of temperature at the time of departure were sought but not found; neither are the deviations from the expected values related to the teneral time itself, so that there do not appear to be any accumulative large errors nor any other behaviour factors seriously affecting time of flight. The experiments in which behaviour is believed to be a major factor are presented in Table 3. These cannot conveniently be classified and require individual examination.

Table 3. *Condensed data for experiments in which aphids are delayed by behaviour*

Exp. no.	Moulting times, G.M.T.			No. aphids	Thermal constant reached at	Expected mean times of flight	Flighting times, G.M.T.		
	Distribution limits		Mean				Distribution limits		Mean
	From	To					From	To	
4	06.15	11.00	07.9/7	41	17.00/7	04.30/8	14.30	10.30	05.3/8
5	16.30	19.00	17.9/7	24	11.00/8	11.00/8	06.15	16.45	12.6/8
8	07.15	09.15	08.3/9	13	18.00/9	07.00/10	07.00	10.00	08.5/10
10	08.15	11.15	09.3/9	33	18.30/9	07.00/10	06.15	11.45	08.4/10
11	09.15	11.30	10.4/9	21	19.30/9	07.00/10	17.30	10.00	07.8/10
15*	08.15	12.30	09.5/10	17	18.30/10	07.00/11	06.15	13.30	08.0/11
18	04.15	10.15	07.2/11	41	19.00/11	09.30/12	16.00	16.15	09.6/12
19	06.15	10.15	08.2/11	66	20.00/11	09.30/12	17.00	15.00	09.3/12
25	05.15	09.45	06.3/13	31	19.30/13	08.30/14	17.00	14.30	08.4/14
31*	05.15	09.15	07.1/16	46	05.30/17	08.30/17	08.00	13.15	08.6/17
34	05.15	09.15	07.1/17	69	17.00/17	08.30/18	08.30	19.15	09.0/18
37*	06.15	11.00	08.6/18	33	03.00/19	06.00/19	18.00	18.30	06.5/19
38*	06.15	11.00	08.6/18	32	03.00/19	06.00/19	14.30	14.30	07.4/19
42	07.15	14.30	09.8/19	29	19.00/19	07.00/20	17.00	11.30	06.7/20

* Mode used, not mean.

In Exp. 4 the thermal constant is reached at 17.00 hr., yet flight did not occur that evening. It may have been dark earlier than usual, but the temperature never fell below 16° C. all night. This is the only occasion when the cause of delay is not evident. Flight took place as soon as it was sufficiently light, at 05.30 hr., for the mean cannot be expected to occur immediately the inhibiting bar is passed.

In Exp. 5 the thermal constant is reached at 11.00 hr. and flight is delayed for $1\frac{1}{2}$ hr. This may be hardly significantly different from the expected time, but inspection of the temperature table shows that temperature was low (*c.* 17° C.) during the morning, and those insects which should have matured and flown before the mean were probably slightly delayed, so that all went after the expected mean time. Exps. 4 and 5 both include aphids from four leaves on which the departure records are rather difficult to interpret (see original data).

Exps. 8, 10 and 15 are the second-day batch of experiments which departed on 2 days, and, as explained earlier (p. 194), these would be expected to have means slightly delayed. Delay of 1 hr. after the expected departure time would cause the

mean to be after 'dark', as are Exps. 11, 18, 19, 25, 37, 38 and 42. All these batches were delayed overnight by darkness and in the morning by low temperature. In col. 6 the expected time of flight is the end of the first half-hour period after the 16° C. bar is passed; they all departed within reasonably short intervals of this time.

Exp. 31 was delayed in the morning from 05.00 to 08.30 hr. by low temperature and departed almost immediately the temperature rose. Exp. 34 was expected to terminate at 17.00 hr. on an evening following rain at 15.00 hr. and of low temperature (*c.* 17° C.); it was also probably dark. The insects departed at the expected time next morning.

CONFIRMATION OF RESULTS

In the field

In addition to experiments given in full in Table 2 of Johnson *et al.* (1957), there was a short series of ten experiments, designed to find out when insects moulting during the night would fly. All alatae were removed from the experimental leaves at dusk, and those which appeared by the next morning were marked then and their flighting times recorded as in the other experiments. It is thus not possible to obtain a mean moulting time for these with any accuracy, for most moulting probably occurred towards the beginning or the end of the moulting period, not in the middle of the night where the mean would be. It is possible, however, to use these experiments as a check for the other data by working back from the mean departure time to find an expected moulting time; only in Exp. A is there any apparent delay due to behaviour. These results are presented in Table 4, from which it will be seen that, out of the ten experiments, in all save A the expected moulting mean falls within the recorded times. In Exp. A the error may not be more than an hour, and this is associated with the fact that the mean flighting time falls outside the known limits of light intensity for flight, i.e. at 21.00 hr., because of the equally balanced departure before and after nightfall.

Allowing a slight variation of the 16° C. bar to flight for individual aphids and some variation of temperature within the crop, it therefore seems that the conditions

Table 4. *Confirmatory experiments in the field*

Exp. no.	Flighting times, G.M.T.			No. aphids	Expected mean moulting time	Moulting times, G.M.T.	
	Distribution limits		Mean			Distribution limits	
	From	To				From	To
A	17.15/8	11.30/9	21.0/8	21	07.30/8	19.00/7	07.10/8
B	11.45/9	17.00/9	14.5/9	48	19.00/8	17.30/8	06.30/9
C	10.00/10	17.00/10	13.3/10	50	19.00/9	17.00/9	06.30/10
D	09.25/13	11.05/14	18.5/13	68	01.00/13	18.30/12	06.15/13
E	19.45/14	19.00/15	09.7/15	32	22.00/13	19.05/13	07.00/15
F	07.15/15	14.15/15	09.5/15	30	22.00/14	19.30/14	07.30/15
G	12.00/19	18.40/19	14.6/19	21	23.00/18	18.30/18	06.00/19
H	14.30/19	19.15/19	16.7/19	52	06.30/19	19.10/18	07.30/19
I	10.45/20	15.30/20	13.1/20	20	19.00/19	18.15/19	07.00/20
J	09.15/21	18.00/21	12.0/21	32	20.30/20	19.15/20	07.00/21

stated in the introduction (p. 190) can account for almost all the variation in teneral period found, with the possibility that some occasional, unsuspected factor may produce a small deviation (Exps. 4, 5 and A).

In the laboratory

To find out if the absolute level of the temperature-velocity curve, with temperatures measured at 1 ft. from the ground, bore any relation to the direct response curve of aphids in relation to the temperature of their immediate surroundings, and also to check the shape of the curve against that obtained at constant temperatures, teneral period was measured at several constant (and some variable) temperatures in the laboratory using the experience gained in the field as a guide. A freehand curve of a shape similar to the logistic, but curving down to zero at the upper limit, was drawn through the constant-temperature means, and this curve was used to calculate the means for the variable temperatures. I have called this a semi-logistic curve.

For this purpose final instar nymphs of alienicolae from culture or from an infested crop were placed on separate leaves standing in water and, as they moulted, were transferred to the constant-temperature cabinet, where they were covered individually by transparent cages. The bulb of the same thermograph as had been used in the field experiments lay between the cages. The accuracy would therefore be of the same order as that obtained in the other experiments, i.e. errors of the order of up to $\pm 0{\cdot}5^\circ$ C. were possible. Variable temperatures were obtained by keeping some of the aphids longer in the laboratory, where the air temperature adjacent to the cages was measured by thermometer, before placing them in the constant-temperature cabinet. The cabinet was illuminated by mercury vapour lamp giving about 200 f.c. at the level of the cages, and this also contributed to raising the temperature. The insects were inspected through a window in the cabinet to obtain the time of flight.

The insects in the lowest temperature group were kept at a temperature below flight threshold until they were estimated to have almost completed their development; then the light was switched on and the temperature raised to about 20° C. (i.e. above the take-off threshold). This is treated as a constant-temperature group as the time at the higher temperature was negligible. Owing to a premature raising of temperature most of the insects had flown before inspection on this occasion, and the plotted mean was obtained from the remaining insects assuming the frequency distribution to be the same as for other groups.

The semi-logistic curve is shown in Fig. 6 along with the means obtained; the logistic curve from the field data is also given for comparison. The close similarity over the rising part of the curve is obvious, and this confirms the conclusions drawn earlier in the paper. The variation of individuals is given in Table 5. The range is approximately $\pm 30\%$ (95% limits) and is a constant proportion at all temperatures, as would be expected if temperature levels merely act through the genetic structure of the population in relation to rate of development, other factors being randomly distributed.

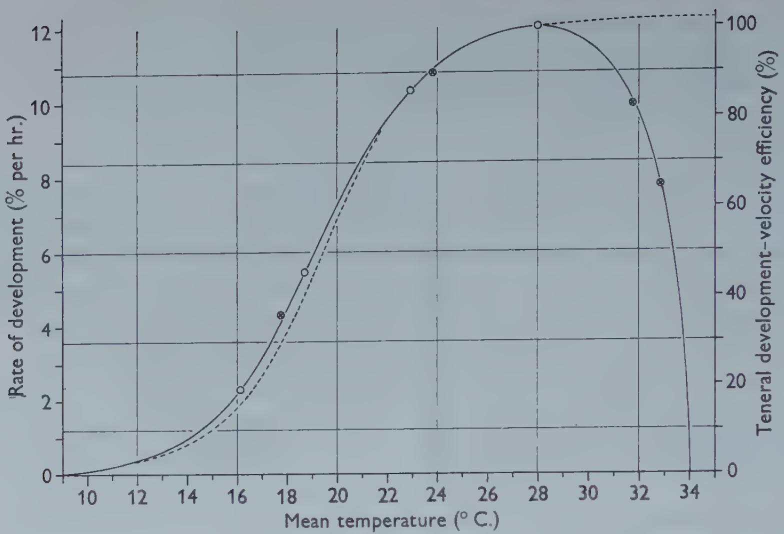

Fig. 6. Semi-logistic temperature relation measured in laboratory compared with logistic curve from nature. ——, curve from laboratory data; - - - -, curve from field data; ○, constant temperature means; ⊗, variable temperature means.

Table 5. *Teneral time of* Aphis fabae *in the laboratory*

No. of aphids	Mean temp. (° C.)	Temp. range (° C.)	Mean developmental rate	Mean teneral time, hours	Standard deviation of time	Standard deviation as % of mean (±)
19	32·9	28–36	7·94	12·61	1·90	15·08
22	31·7	17–32	9·90	10·10	1·89	18·71
31	28·0	Const.	12·10	8·26	1·63	19·73
19	23·8	17–31	10·80	9·26	1·03	11·12
44	22·9	Const.	10·23	9·78	1·22	12·47
25	18·8	Const.	5·62	17·81	2·43	13·64
35	17·7	15–32	4·33	23·12	3·03	13·10
(15)	16·1	Const.	(2·39)	(41·8)	—	—

THE TENERAL STAGE

Although the importance of flight to insects has been well emphasized, the time of acquisition of wings has received remarkably little attention. This may be attributed partly to the apparent rapidity of the process in some species, especially those with large wings in which the actual expansion of the wings is usually completed in a few minutes. Interest has also been directed toward the change in colour which often takes place; the time involved in this process, however, may be of quite a different order. For example, in Odonata complete coloration may take several weeks (Tillyard, 1917, p. 98). and the same may apply to Coleoptera, e.g. 25 days in *Leptinotarsa decemlineata* Say (Dunn, 1951). The actual process of cuticle reformation may take an equally long time, e.g. 2 weeks in adults of *Rhodnius prolixus* Stål (Wigglesworth & Gillett, 1936). But these insects have resumed active life before this.

There is, however, an interval succeeding any ecdysis during which the soft cuticle and relative inactivity of the insect are likely to render it particularly vulnerable. It is difficult to obtain evidence of this in nature although it justifies further examination, but Corbet (1956) ascribed a loss of 35·4% of a culture of *Anax imperator* Leach to cannibalism *during ecdysis*. It is this vulnerable, inactive stage which is of particular ecological interest, and which is terminated by resumption of active life, viz. by flight, after eclosion of the imago.

If flight normally occurs as soon as it is possible, then a state of flight maturity is implied and the stage preceding it is developmental. This is justified for *Aphis fabae* by the results presented here. The word 'teneral' (Latin *tener*) means literally 'soft' or 'immature' and aptly describes the stage up to flight maturity, although pigmentation maturity has been taken as the criterion in Odonata.

The changes in wing appearance and pigmentation after eclosion in *A. fabae* were examined in relation to flight by B. Johnson (1955), who found that the wings passed through five recognizable stages: opaque; transparent with a shiny white appearance; with a dull blue irridescence; with a reddish sheen and looking much harder in texture; finally, the formation of a pigmented bar along the post-axial border of the radial sector within 'several hours' of the end of the teneral period. The 'red' stage is reached 'at or shortly before reaching the end of the teneral period'; at this stage the aphid will take-off if disturbed, and he described them as 'flight mature'. He pointed out that they did not appear to fly immediately this condition was reached, and Moericke (1955) has emphasized this as a behaviour inhibition (*verhaltensmässige*), the insects not being in a 'mood' for flight. But from the results presented here it would appear that this delay is directly related to temperature, for it is included in the teneral time; it cannot therefore be in the nature of a behaviour inhibition unless it is included in the individual variation around the mean departure time, and in this respect it is so short as to be negligible.

That the time interval is short is shown by B. Johnson's own description of aphids caught in a suction trap on the same plot on which these experiments were carried out. Of 247 aphids 238 were freshly emerged with no pigmentation of the radial sector. This confirms the fact that *A. fabae* flies almost as soon as it is capable of flight, at least in the field, and that this is therefore a good criterion for termination of the teneral stage.

The behaviour of aphids in the laboratory during the teneral period is very consistent. Immediately after moulting they walk freely to a suitably sheltered place and settle down. At this time they are easily disturbed and quickly respond to visual and tactile stimuli. Once they have settled down the wings rapidly expand, the stylets are inserted and the aphids then remain in an almost torpid state. During this time light intensity and touch have a very much reduced effect in inducing movement. After sitting motionless for hours the insects suddenly withdraw their stylets, wave their antennae and walk up the leaf on which they have been sitting and possibly feeding. They then walk about, at a speed dependent upon the temperature, until they find a suitable take-off point on the leaf, and within a few seconds fly away. As an example, at 29° C. one aphid took 50 sec. from withdrawl of the stylets

to flight, and whilst this observation was being made another aphid had gone—in an even shorter interval. At lower temperatures the time taken is correspondingly greater.

It was at first thought that this immediate, unstimulated, flight response of *A. fabae* at the end of the teneral period might be a migratory act, and to confirm this, pupae of the hibernating, non-migratory, autumn phase of *Aglais urticae* L. (Lepidoptera), the Small Tortoiseshell butterfly, were timed and observed at eclosion in different temperatures. The results do not cover a sufficiently wide range to give a full semi-logistic response curve, but the times recorded in Table 6 show essentially the same picture as for aphids. The behaviour also conformed remarkably well to the description for *Aphis fabae* given above, except that the insect coils and uncoils its proboscis at the time when the aphid withdraws its stylets.

Table 6. *Teneral time at different temperatures in* Aglais urticae *L.* (*Lepidoptera*)

Temperature (° C.)	17·4	18·5	18·8	19·9	20·8	22·8	24·1	26·8	31·8
No. of insects	10	10	10	10	10	10	10	10	7
Mean teneral time in hours	4·7	4·5	3·8	2·9	2·6	2·6	2·3	1·8	1·3

It seems that this obligatory waiting period may be temperature-dependent in groups other than aphids, and that the use of the word 'teneral' in this connexion may have application outside the Aphididae.

Glossina spp., after eclosion, pass through an active, but wingless, 'spider' stage which lasts for up to an hour, and then after expansion of the wings may be described as 'inactive' for the first 2 days (Jackson, 1946). The 'teneral' stage is described as terminated by the closure of the ptilinal suture, except in *G. palpalis fuscipes* in which the suture never closes; but this depends upon the first blood meal to harden the cuticle (Jackson, 1945). Thus the 'teneral' stage would be extended by failure to find food and is not, in that case, development-limited. Buxton (1955) referred particularly to the 'spider' stage as vulnerable, but this probably applies to the inactive stage also, and this may be temperature-dependent as in *Aphis fabae* and *Aglais urticae*, the spider stage corresponding with the shorter interval which these species spend in finding a suitable site to settle down.

The term 'spider stage' could quite well be applied particularly to *Aglais urticae* at that time, and it may be that continued activity defers further development as in the 'walker' stage (Graham-Smith, 1916) of *Calliphora* (Diptera) forced to continue digging in the soil (Fraenkel, 1935). This will not be of general occurrence in wild populations, and in considering teneral time, whatever definition is used, temperature will be a major factor and should be stated.

Whether or not aphids feed during this time is not known, but insects passing their teneral stage on leaves did not move if the leaves wilted, nor did those few on the cage wall depart appreciably from the general pattern of response. Moericke (1955) found a slight reduction, 1–1½ hr, in the teneral time of 'hungry' *Myzus*

persicae Sulz. It would be difficult, without very large samples, to verify this, especially at temperatures other than optimal, since it is well within the standard deviation and it is not known if temperature was taken into consideration.

SUMMARY

1. The teneral stage in *Aphis fabae* Scop. is defined as the interval between eclosion of the alate imago and flight when flight is not extrinsically activated or inhibited.

2. In field marking experiments the teneral stage is shown to be usually development-limited, not behaviour-limited, by its relationship to temperature.

3. It is suggested that the logistic curve fits the rising temperature × development-velocity curve because this is a statistical distribution like the Pearson type 1.

4. The logistic curve is fitted to teneral period in naturally varying temperatures by a method of successive approximation.

5. In trapping experiments take-off is inhibited by light below *c.* 100 f.c. and by temperature below *c.* 16° C. at 1 ft. in a bean crop.

6. These behaviour responses sometimes lengthen the apparent teneral period.

7. The results are confirmed in the laboratory at constant and at varying temperatures.

8. This also confirms that temperature changes *per se* even over a range of *c.* 7–34° C., and other climatic factors, have negligible effect on rate of development during the teneral period.

9. Individual variation is a constant proportion of teneral time at all temperatures.

10. In laboratory experiments the same criteria apply to *Aglais urticae* L.

11. This period of inactivity warrants further examination in an ecological context.

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REFERENCES

ANDREWARTHA, H. G. (1944). Air temperature records as a guide to the date of hatching of the nymphs of *Austroicetes cruciata* Sauss. (Orthoptera). *Bull. Ent. Res.* **35**, 31–41.

BROADBENT, L. (1949). Factors affecting the activity of alatae of the aphids *Myzus persicae* Sulzer and *Brevicoryne brassicae* L. *Ann. Appl. Biol.* **36**, 40–62.

BUXTON, P. A. (1955). *The Natural History of Tsetse Flies.* London: H. K. Lewis.

CORBET, P. S. (1956). Environmental factors influencing the induction and termination of diapause in the emperor dragonfly, *Anax imperator* Leach (Odonata: Aeshnidae). *J. Exp. Biol.* **33**, 1–14.

DAVIDSON, J. (1942). On the speed of development of eggs at constant temperatures. *Aust. J. Exp. Biol.* **20**, 233–9.

DAVIDSON, J. (1944). On the relationship between temperature and rate of development of insects at constant temperatures. *J. Anim. Ecol.* **13**, 26–38.

DUNN, E. (1951). Wing coloration as a means of determining the age of the Colorado beetle (*Leptinotarsa decemlineata* Say). *Ann. Appl. Biol.* **38**, 433–4.

ELDERTON, W. P. (1953). *Frequency Curves and Correlation*, 4th ed. Cambridge University Press.

FRAENKEL, G. (1935). Observations and experiments on the blow-fly (*Calliphora erythrocephala*) during the first day after emergence. *Proc. Zool. Soc. Lond.* pp. 893–904.

GRAHAM-SMITH, G. S. (1916). Observations on the habits and parasites of common flies. *Parasitology*, **8**, 440–544.

Haine, E. (1956). Häutung, Abflug und Landung der Blattläuse in Wechselwirkung auf die Blattlauszahlen in der Luft. *Mitt. biol. Bundesanst. Berl.* **85**, 23–7.

Jackson, C. H. N. (1945). Comparative studies of the habitat requirements of tsetse fly species. *J. Anim. Ecol.* **14**, 46–51.

Jackson, C. H. N. (1946). An artificially isolated generation of tsetse flies (Diptera). *Bull. Ent. Res.* **37**, 291–9.

Johnson, B. (1955). Studies in flight, host selection and reproduction in aphids. Thesis (Ph.D.), London University.

Johnson, C. G. (1952). The changing numbers of *Aphis fabae* Scop. flying at crop level, in relation to current weather and to the population on the crop. *Ann. Appl. Biol.* **39**, 525–47.

Johnson, C. G & Taylor, L. R. (1955). The measurement of insect density in the air. *Lab. Pract.* **4**, 187–92, 235–9.

Johnson, C. G. & Taylor, L. R. (1957). Periodism and energy summation with special reference to flight rhythms in aphids. *J. Exp. Biol.* **34**, 209–21.

Johnson, C. G., Taylor, L. R. & Haine, E. (1957). Analysis and reconstruction of diurnal flight curves in alienicolae of *Aphis fabae* Scop. *Ann. Appl. Biol.* (in the Press).

Moericke, V. (1955). Über die Lebensgewohnheiten der geflugelten Blattläuse (*Aphidina*) unter besonderer Berücksichtigung des Verhaltens bein Landen. *Z. angew. Ent.* **37**, 29–91.

Taylor, L. R. (1951). An improved suction trap for insects. *Ann. Appl. Biol.* **38**, 582–91.

Taylor, L. R. (1955). The standardization of air-flow in insect suction traps. *Ann. Appl. Biol.* **43**, 390–408.

Tillyard, R. J. (1917). *The Biology of Dragonflies.* Cambridge Zoological Series.

Verworn, M. (1894). *Allgemeine Physiologie.* Jena.

Wigglesworth, V. B. & Gillett, J. D. (1936). The loss of water during ecdysis in *Rhodnius prolixus* Stål (Hemiptera). *Proc. R. Ent. Soc. Lond.* A, **11**, 104–7.

PERIODISM AND ENERGY SUMMATION WITH SPECIAL REFERENCE TO FLIGHT RHYTHMS IN APHIDS

By C. G. JOHNSON and L. R. TAYLOR
Department of Entomology, Rothamsted Experimental Station, Harpenden, Herts

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INTRODUCTION

Diurnal rhythms, as in insect flight or in plant movement, are often due to the periodic repetition of an act by the same individual. Some organisms, however, perform an act only once in their lives (such as hatching from the egg), yet this single act repeated by different individuals is synchronized to show a periodicity in the population. Here lies a fundamental difference between population periodicity *per se*, which can never occur in an individual, and individual periodicity which is reflected in populations.

But periodic acts by the same individuals and single acts by a succession of different individuals both require synchronization for a periodicity to be manifested in the population. This synchronization of the only act on the one hand and of the initial act on the other, may be due to the same cause; namely, to a differential rate of development preceding the act which, in different individuals, brings the acts together.

In the same way, it is possible also to regard periodic behaviour in a single organism as due to the periodic changes in the rates of its developmental processes.

This general principle is applied in this paper to the daily bimodal flight rhythms in populations of *Aphis fabae* Scop.

INITIAL FLIGHT IN APHIDS

Winged aphids, produced on a crop, fly away when only a few hours old, never to return; the action is not repeated.

Nevertheless, a bimodal curve of numbers departing during the day (Fig. 1) with no flight at night is a common feature of such populations (Johnson, 1954). It is shown in this paper that such a periodicity is primarily due to the alternate contraction and lengthening of the period of maturation preceding flight (the teneral period) as the temperature during the day and night fluctuates. A limited number of rather simplified examples is given; they do not cover all possible variations on the general pattern. For example, on different days the two peaks may differ widely in relative height, amplitude and time of occurrence; one may be suppressed or merged into the other. Such variations depend on the temperature and on the organisms' relations to it; they are of interest mainly to specialists and will be further analysed for aphids elsewhere (Johnson, Taylor & Haine, 1957).

THE RECONSTRUCTION OF SINGLE-PEAKED AND BIMODAL CURVES

There are three elements contributing to the periodicity of flight in aphid populations. These are: the rate of moulting into the winged adults; the length of the teneral period from moulting to flight; the environmental factors inhibiting flight.

All these elements vary in a complex way especially with temperature; and in nature they are integrated to produce a flight curve. In order to illustrate this process and the method for reconstructing flight curves from the variable and complex data obtained in the field it is convenient first to consider a simplified example (case 1) to bring out the basic interactions between the above three elements in relation to temperature.

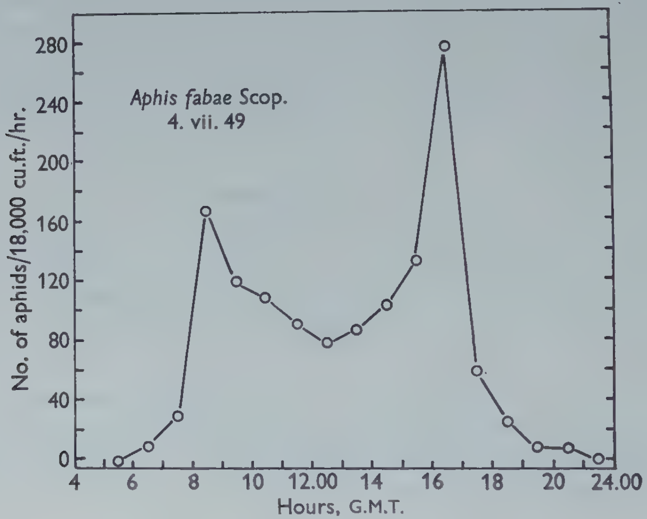

Fig. 1. Typical curve of aerial density change for aliencolae of *A. fabae* flying above a bean crop on which they were produced.

Case 1

In temperate countries the mean hourly temperature during the 24 hr. often somewhat resembles a sine curve. Consider therefore a simple example (Fig. 2) in which the temperature during the day and night follows a sinusoidal curve ($\theta_1\theta_6$) which is repeated on two successive days and never falls below a developmental threshold of 0° C. on the empirical temperature scale; the moulting rate is constant at 10 per hr. and the velocity of development during the teneral period is linearly related to temperature (see pp. 213 and 215). Flight occurs immediately after maturation.

In Fig. 2 an organism moulted at time t_1 and temperature θ_1 matures and flies at time t_2 and temperature θ_2. The amount of heat necessary to complete development is represented by the sum of all the temperatures for the period of development;

that is, by the area under the sine curve between moulting and flight. This is the thermal constant:

$$\int_{\theta_1}^{\theta_2} \theta \, dt = \sum_{t_1}^{t_2} \theta = \text{constant.}$$

The teneral period $(t_2 - t_1) = \sum_{t_1}^{t_2} \theta / \bar{\theta}$. But because $\bar{\theta}$, the mean temperature, varies with time of moulting, and therefore with θ_1, so $\sum_{t_1}^{t_2} \theta / \bar{\theta}$, the teneral period, will also vary with the time of moulting.

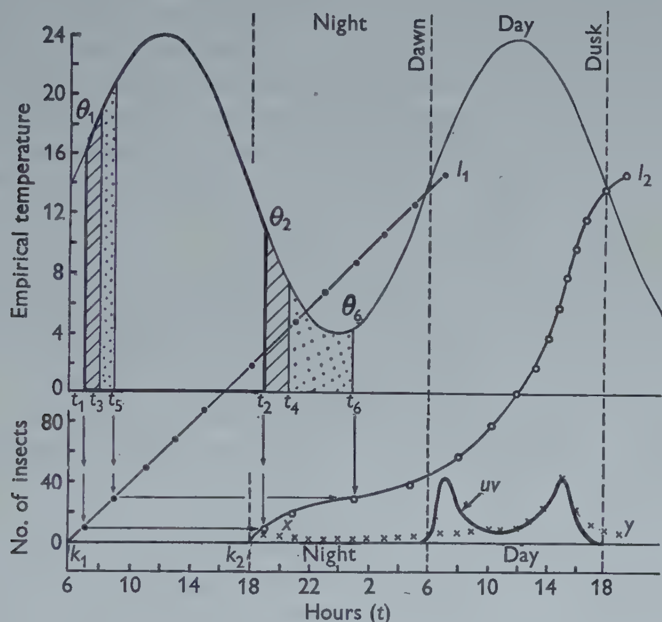

Fig. 2. Theoretical reconstruction of bimodal flight curve. $\theta_1\theta_6$, temperature curve; t_1t_2, t_3t_4, t_5t_6, teneral periods of different length; k_1l_1, accumulative moulting rate, constant at 10 per hr.; k_2l_2, accumulative maturation curve; xy, hourly differences along k_2l_2 showing number of insects maturing each hour; uv, flight curve showing number of insects departing each hour.

An area, for moulting at t_1 and maturation at t_2, is heavily outlined on the sine curve in Fig. 2. For moulting 1 hr. later at t_3, the cross-hatched area is subtracted from the left and added on to the right-hand side of the original area, so maintaining a constant area but shifting the maturation time relatively farther to the right. Similarly for moulting at t_5 (dotted area) and so on. Thus as the temperature at the time of moulting moves along the sine curve, so the period of development, t_1t_2, t_3t_4, t_5t_6, etc., also shortens and lengthens rhythmically as the mean temperature rises and falls.

As each successive batch of ten insects moults at mean intervals of 1 hr., so they mature at longer or shorter intervals according to the mean temperature. The moulting rate, with the accumulated number of moults along curve k_1l_1 (Fig. 2) is

thus translated to a curve k_2l_2 for accumulated mature insects. If the insect flies off immediately on maturation then the numbers departing each hour will be the successive hourly differences along the accumulated maturation curve k_2l_2; this is represented by a single-peaked curve, xy.

The manner in which the single-peaked curve, xy, is produced, however, is applicable to any energy-controlled developmental process (whether in a population or an individual) where the energy fluctuates and complete development is attained approximately in a single cycle.

However, flight in aphids may be inhibited by factors such as light intensity and temperature. For example, aphids do not fly at night and, if completing their development during the night, will wait until dawn when they should depart, not all at once, but distributed over a certain period. These would produce a first peak in the morning in addition to the second, later peak. Also as light diminishes at night the rate of take-off also diminishes serially; arbitrary values of 75 and 50% for the take-off of mature individuals have therefore been given respectively for the 2 and 1 hr. preceding sunset in case 1. The individuals remaining, though mature, would not fly; they are added on to the next morning peak. The final result is a double-peaked curve, the heavy line uv in Fig. 2.

This is the theoretical basis for the curve of bimodal flight in aphid populations. Obviously the shape of the accumulative maturation curve k_2l_2 and thus of the first differential or maturation rate curve, xy, will depend on the extent of the constant area under the temperature curve; that is, it will depend on the thermal constant of the organism.

It is now possible to analyse the more complex process which actually occurs with *A. fabae* in nature.

THE FLIGHT OF *APHIS FABAE* ON 6 JULY 1952

In July 1952 a suction trap (Taylor, 1951) was operated in a bean crop heavily infested with *A. fabae*, measuring the aerial aphid density (C. G. Johnson & Taylor, 1955), and on 6 July showed a typical bimodal curve (Fig. 4*c*); the greater proportion of all aphids caught by this trap had just left the crop on their first flight (see p. 213).

Temperature was recorded continuously in the crop and was always above the threshold for the maturation of winged aphids. The rate at which alatae were produced by moulting (the moulting rate, case 1) was also recorded approximately once an hour. A complete description of the experimental procedure is given elsewhere (Johnson, Haine & Cockbain, 1957; Johnson, Taylor & Haine, 1957).

The natural example with alienicolae of *A. fabae* in summer differs from the formalized example in case 1, in the following ways.

Temperature

The temperature curve is not sinusoidal, but steeper on the ascent than on the descent; there are slight irregularities from hour to hour, and it passes above the developmental optimum of 28° C. (Fig. 3).

Moulting rate

Winged aphids are produced when the 4th larval instar moults. In nature the moulting rate is not constant as in case 1 but has a well-marked periodicity of its own (Johnson, Haine & Cockbain, 1957).

Developmental period

After moulting from the 4th larval instar, the alate adult passes through a period of maturation (the teneral period) before flight. This takes several hours.

The length of the teneral period varies with temperature, in the manner typical of most developmental processes; its threshold may be taken as 9° C. and its optimum is 28° C. The development-velocity curve against temperature is semi-logistic, not linear as in case 1 (see p. 210). A full description of the temperature relations of the teneral period is given in Taylor (1957), and temperature is the only factor we need consider as affecting the duration of the teneral period.

Flight inhibitors

In nature the aphid flies away immediately at the end of the teneral period (indeed this may be used to define its length) only if weather and light permit.

Light. Experience in the field shows that approximately 1–2 hr. after sunrise or 1–2 hr. before sunset may be regarded as the equivalent of the light threshold for take-off for the purposes of this paper (see p. 212).

Temperature. Aphids begin to take off when the temperature rises above a threshold. In this example the temperature threshold comes after the light threshold has been passed both in the morning and in the evening; this is typical. We may therefore regard temperature as the effective releaser to flight in the morning and light, 1–2 hr. before sunset, as the inhibitor in the evening.

The threshold for aphid take-off was determined both in the field and in the laboratory; the lowest temperature at which any aphid was seen to take off was 15·5° C. But the lowest temperature for take-off, like any other attribute of an organism, is not the same for each individual nor perhaps for the same individual on different occasions; and this attribute will be distributed about a mean value.

Provided the temperature is always above the upper limit of this distribution any aphid will take off as with a take-off after landing from a previous flight. But when the temperature is rising through the range covered by the distribution and we are considering initial take-off, then a few aphids will take off at 15·5° C., more at 16·5° C. and so on until the optimum is reached, after which there will be a decline in the initial take-off rate. As the remarks above indicate this does not imply an inhibition of take-off above the upper limit of the distribution.

The frequency distribution of take-off obtained in the laboratory is slightly skew, with a mode at approximately 17·3° C. (Table 1). This agrees with a distribution obtained from *A. fabae* in the field by Müller & Unger (1951) in Germany.

These remarks apply to the single act of take-off. Total numbers in active flight will be the sum of the successive numbers taking off (i.e. the integral of the frequency

distribution) and, rising to a maximum, will be followed by a plateau until landing produces a decline in the numbers flying. This gives the familiar activity curve which may be confused with the threshold distribution.

In nature the trap samples aphids as they leave the crop and so reflects the take-off rate. Consequently the first peak of flight is a frequency distribution of take-off.

Table 1. *Frequency distribution of a batch of* Aphis fabae *taking flight at temperatures rising past the flight threshold*

Values fitted to log (temperature − 11·5° C.) by the normal probability function (table in Pearson & Hartley, 1954).

° C.	No. of aphids	Fitted values
15·25	0	0·5
15·75	3	3·3
16·25	11	10·9
16·75	26	20·4
17·25	19	25·0
17·75	25	22·1
18·25	17	15·4
18·75	4	8·8
19·25	8	4·3
19·75	3	1·9
20·25	0	0·7

Max. take-off (mode) at 17·3° C.

The frequency distribution of take-off in a batch of *A. fabae* with the temperature rising slowly past the threshold is shown. Alatae of *A. fabae* were kept in the dark at laboratory temperatures and about 24 hr. later on the following morning were allowed to take off from bean leaves in daylight on a laboratory bench; the temperature was recorded as each aphid took flight. The fitted distribution is

$$y = y_0 \exp\left[-111{\cdot}111\,\{\log_{10}(\theta - 11{\cdot}5) - 0{\cdot}7632\}^2\right],$$

where y = take-off rate at temperature θ° C. y_0 = take-off rate at the mode (17·3° C.).

Wind speed. A moderate wind blowing directly on an aphid delays take-off but does not prevent it, for the aphid tries repeatedly, eventually taking-off in spite of the wind (Haine, 1955*a*, *b*). The experiments on 5–6 July 1952 were made in a sheltered garden and the effect of wind speed in delaying take-off can be neglected (Taylor, 1957); this view is also justified by the accuracy of the reconstructed curves in this paper (Fig. 4).

To sum up therefore, it may be said that the aphids in this example take flight immediately they are flight-mature except in the early morning before the temperature has risen to 15·5° C. and after 1–2 hr. before sunset; and that this is typical of summer conditions in the south of England.

Reconstruction of the flight curve for 6 July 1952

Given the moulting rates and the temperatures throughout the night and day of 5 and 6 July (Fig. 3), together with the flight thresholds and the temperature velocity curve for the teneral period (Taylor, 1957), it is possible to reconstruct an expected

flight curve for 6 July using the method described in case 1. This can then be matched with the actual observed flight curve (Fig. 4) obtained from the suction trap in the same crop and on the same occasion.

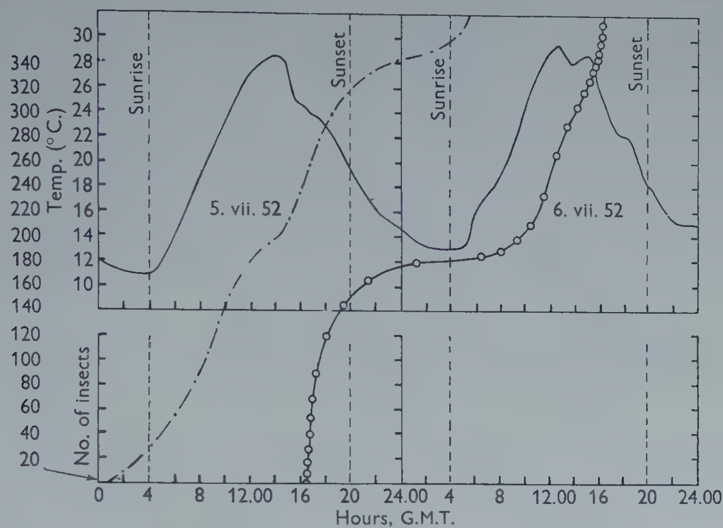

Fig. 3. Basic data for reconstruction of flight curves of 6 July 1952 (cases 2 and 3). —, temperature; ·—·, observed accumulative moulting rate. This curve is smoothed by taking the 3 hr. running means from original data (see text); o—o, calculated accumulative maturation curve from which flight curves in Fig. 4*a*, *b* are obtained.

Thermal summation for 6 July 1952

Each hour of the teneral period spent at a certain mean temperature adds an increment to development. In case 1 the temperature × development-velocity curve was linear, and an hour at any temperature added the same increment; the empirical temperature curve itself could therefore be used for summation. The temperature-velocity curve for the teneral period in nature is semi-logistic, and an hour at different temperatures adds different increments; a certain developmental increment must therefore be assigned specifically to the mean temperature for each hour during a day. In other words the actual temperature curve must be transformed into a curve for developmental increments; we have called this the temperature-equivalent curve (Table 2, Taylor, 1957), and it is used in the constructions shown in Fig. 4*a*, *b*. The actual temperature curve is shown in Fig. 3.

Case 2

It is advisable first to construct an expected flight curve based on a constant moulting rate.

This shows that in spite of departures of temperature from a sine curve, the use of a semi-logistic temperature-velocity curve, the inclusion of a temperature threshold for take-off and in spite of other out-of-doors conditions, the basic bimodality is established as in case 1 (compare Fig. 4*a*, *c*).

Case 3

A second curve reconstructed with the variable moulting rate observed on 5–6 July (Fig. 3) gives a still closer agreement of expected and observed curves (cf. Fig. 4*b*, *c*). Thus the depression in numbers 1300 and 1600 hr. in case 3 is caused by the reduction in moulting overnight. This depression is commonly seen in aphid flight curves (Müller & Unger, 1952).

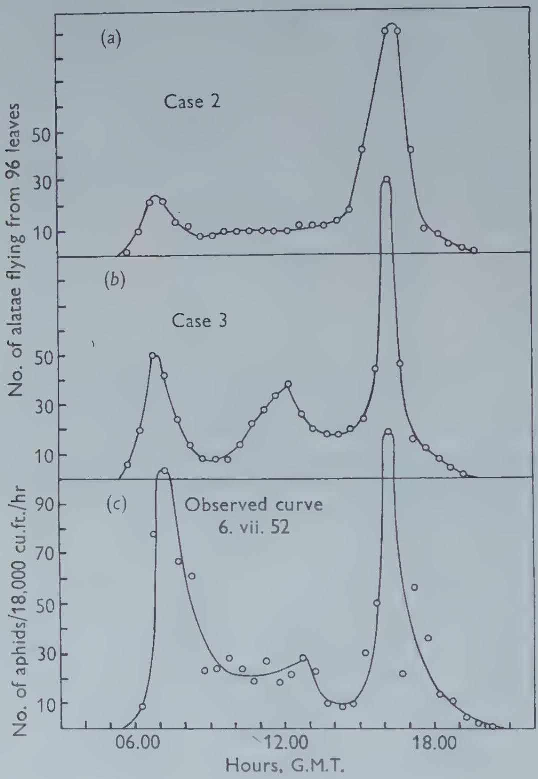

Fig. 4. The observed and expected flight curves of *A. fabae* on 6 July 1952. (*a*) expected, reconstructed curve with constant moulting rate (case 2); (*b*) expected, reconstructed curve with observed, variable moulting rate (case 3); (*c*) observed flight curve: 9 in. suction trap showing aerial density (no aphids per 9000 cu.ft. air per ½ hr.). In (*a*) and (*b*) the numbers flying per hour can only be related to the moulting rate (on 96 leaves) not to aerial density.

There are, however, several discrepancies to consider. The first peak in case 3 (Fig. 4*b*) was obtained by arranging the number of mature insects estimated to have accumulated overnight in a frequency of flight according to a curve fitted to the distribution in Table 1; it is, in fact, identical in form to the observed peak (Fig. 4*c*), but it appears 30 min. too early and is too small. A slight discrepancy in its position is not surprising, for the position is decided by the actual temperatures at that time of the day; these are rising so rapidly that differences between air and leaf temperatures or an incorrect placing of the thermograph could produce such an

effect. The size of the first peak depends also on the contribution made by insects maturing but not flying late on the previous evening; this cannot be estimated satisfactorily at present, on account of errors in estimating moulting rates from one day to another and in the effect of waning light on take-off.

The actual moulting rate was not observed exactly at hourly intervals (for details see Johnson, Haine & Cockbain, 1957); interpolated values at hourly intervals had therefore to be estimated and 3 hr. running means taken to obtain the moulting rate curve in the reconstruction of case 3. The slight depression immediately following the first reconstructed peak is caused by an unusual reduction in the moulting rate of the insects on the sample leaves at about 13·00 hr. on the day before. Such a discrepancy in the fit between observed and expected flight curves in case 3 (Fig. 4), especially for the first peak, is thus not surprising in view of sampling errors in estimating the moulting rate, apart from those of the suction trap itself.

TYPES OF TEMPERATURE CURVE AND THEIR EFFECTS ON PERIODICITY

Variations of the bimodal flight curve of aphids are extremely numerous; they are caused mainly by variation in the daily temperature curve of the same or previous day affecting the duration of the teneral period, in association with a wide variability in moulting rates. Temperature has the greater effect, particularly in the timing of peaks; variation in moulting rate affects the height of peaks and the occurrence of subsidiary peaks.

Two examples are given here to illustrate the kind of modifications likely to occur with temperature curves of different character to those already considered; these two examples are based on actual temperature records observed in the field but with minor variations smoothed out (Fig. 5). A constant moulting rate will be used together with the temperature-velocity curve and flight thresholds for *A. fabae*. In the illustrations to these two examples (Figs. 6, 7), the flight curve differs very little from the hourly maturation curve, and the latter alone is drawn.

Case 4 (Fig. 6)

The mean temperature for 24 hr. is 16·4° C. with a range of 7–24° C. The temperature passes below the developmental threshold for nearly 6 hr. (Fig. 5*a*).

Insects which fail to complete the teneral period in time for the second peak are further delayed in their development by the sub-threshold temperatures at night. Maturation is then completed very rapidly as the temperature rises next morning and the different individuals, all maturing in a short time, fly off as a steep first peak in the morning. The second peak is flattened to virtual extinction.

Thus, if the temperature within one cycle (in this case 24 hr.) does not allow development to be completed (the sum of the increments being less than the thermal constant), the second peak will disappear and become part of the first peak in the next period.

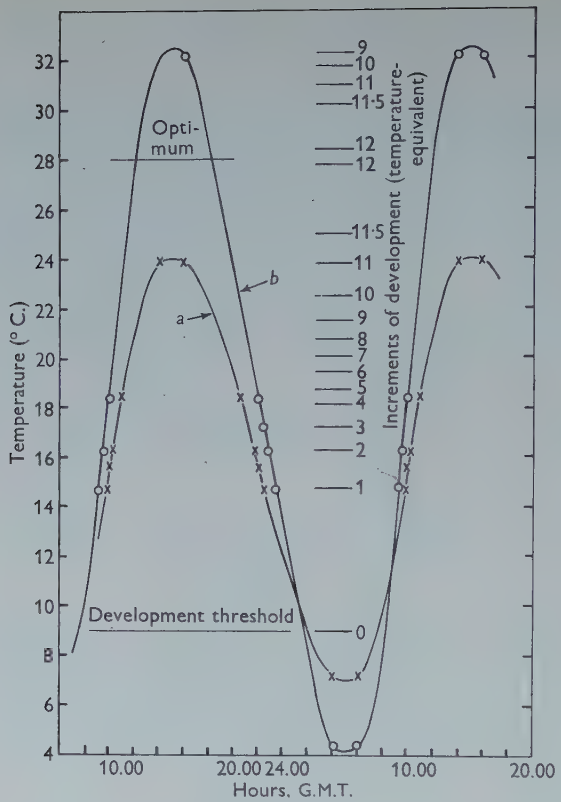

Fig. 5. Two observed, smoothed temperature curves *a* and *b* on which cases 4 and 5 are based respectively.

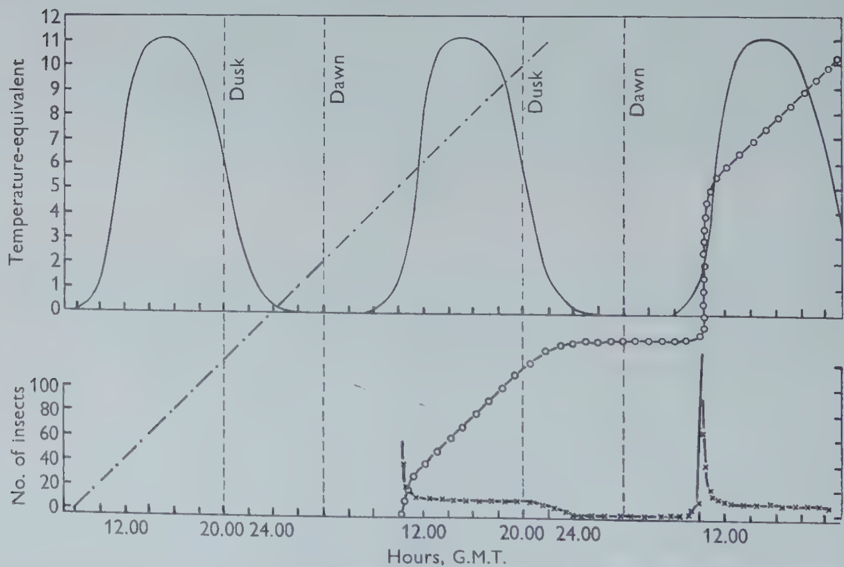

Fig. 6. Reconstructed maturation and flight curves: case 4. —, temperature-equivalent curve: actual temperatures in Fig. 5; · — ·, constant moulting rate (accumulative); o—o, accumulative maturation curve; × — ×, maturation rate per hour: the flight curve is similar to this (see text). The mean temperature of the day is too low for complete maturation: therefore what would normally be the second flight peak is displaced to become the first peak next day.

Case 5 (Fig. 7)

The mean temperature for 24 hr. is 18·2° C. with a range of 4–30·5° C. The temperature passes below the developmental threshold for 6½ hr. and above the optimum for 6 hr.

When the temperature rises above the optimum for teneral development the teneral period is lengthened, and a sufficient rise above the optimum could virtually inhibit development, though this has not been observed in nature. A more common effect is a slight excess of temperature and a retardation of development.

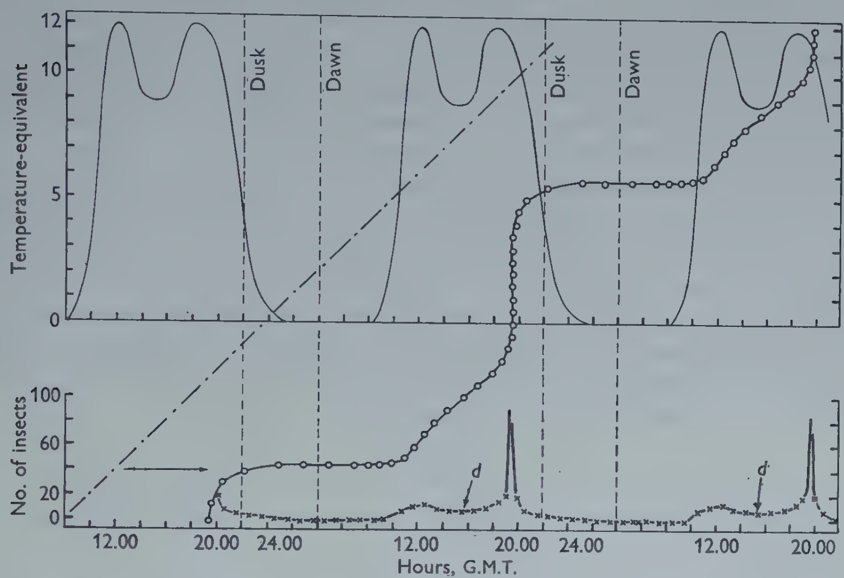

Fig. 7. Reconstructed maturation and flight curves: case 5. Symbols as in Fig. 6. The temperature passes above the optimum and below the threshold (see Fig. 5). The depression *d* is caused by temperatures above the optimum on the preceding day.

If the teneral period is lengthened both by sub-threshold temperatures and by temperatures above the optimum a shift of the second peak into the following day might occur as in case 4. If, however, the mean temperature for the day is also high, so allowing completion of the teneral period within the same day, the second peak will occur normally but the first peak will diminish: this is the effect in case 5.

In cases 1, 4 and 5 it has been assumed that the temperature curves are similar for two successive days. Obviously, modifications will occur when the temperature is different on successive days.

CONCLUSIONS

This paper has considered the specific problem of aphid flight rhythms, but the principles have a general application. The role of differential development rates in conjunction with threshold effects in causing rhythms of other actions, on different time scales and in other organisms, is obvious. It is tacitly accepted in the seasonal

growth of insect populations which has its equivalent in the accumulative maturation curve in Figs. 2, 6 and 7. In seasonal population change it is the reproductive rather than the developmental element which is commonly stressed, but as shown in this paper, changes in reproductive rates (which are equivalent to the moulting rates in Figs. 2, 6 and 7) may only modify a basic pattern due primarily to variable periods of development.

In fact the seasonal and bimodal periodicity of emergence of the dragon fly, *Anax imperator* Leach, between May and July appears to be an example (Corbet, 1954, 1955). There the period for cessation of development during diapause represents the period below the temperature threshold for development. It would seem, however, that the annual temperature curve itself, rather than diapause, might produce the effects observed as an almost exact parallel to case 4 in this paper; as with the dragon fly, case 4 has a very much flattened second peak. There are numerous similar examples throughout the literature.

But apart from seasonal population growth many short-term periodicities which might tacitly be assumed as behavioural in character could be due to a synchronization of development in the individuals of a population and not susceptible to an interpretation primarily in terms of behaviour alone.

This paper has dealt mainly with an insect, but apart from other poikilotherms, where a similar effect is likely, it is theoretically possible that a similar mechanism may apply even in a homoiotherm if a summation of increments with some environmental factor operates on a regulatory mechanism.

SUMMARY

1. Summer populations of *Aphis fabae* often show a bimodal flight curve with no flight at night.

2. The teneral period between moulting and flight depends on temperature and can be estimated.

3. Increase in temperature causes the teneral period to shorten and is followed, some time later, by an increase in rate of take-off.

4. This produces the afternoon peak of flight.

5. The morning peak is usually due to aphids which, maturing overnight, accumulate and fly when rising temperature permits.

6. A graphical method is given for constructing flight curves from constant, or observed, moulting rates and the temperature during the teneral period.

7. Population periodicities in which each individual acts only once, are distinguished from individual periodicities in which the same act is repeated by the same individuals.

8. Synchronization is necessary for either type to be evident, and this may be due to rhythmic fluctuations in developmental increments preceding the act, even in short-term periodicities, rather than to behaviour responses.

9. This may apply to rhythms of flight, as in aphids, or of emergence; to seasonal periodic growth of populations in insects; or to populations of cells in regulatory organs.

REFERENCES

Corbet, P. S. (1954). Seasonal regulation in British dragonflies. *Nature, Lond.*, **174**, 655.

Corbet, P. S. (1955). A critical response to changing length of day in an insect. *Nature, Lond.*, **175**, 338.

Haine, E. (1955*a*). Aphid take-off in controlled wind speeds. *Nature, Lond.*, **175**, 474.

Haine, E. (1955*b*). Beobachtungen über das Flugstartverhalten und die Dauer der Flugfähigkeit der Männchen von *Periphyllus aceris acericola* Wlk. *Anz. Schädlingsk.* **28**, 67–70.

Johnson, C. G. (1954). Aphid migration in relation to weather. *Biol. Rev.* **29**, 87–118.

Johnson, C. G., Haine, E. & Cockbain, A. J. (1957). Moulting rhythms in the alienicolae of *Aphis fabae* Scop. (Hemiptera: Aphididae) in the field. *Ann. Appl. Biol.* (in the Press).

Johnson, C. G. & Taylor, L. R. (1955). The measurement of insect density in the air. *Lab. Pract.* **4**, 187–92, 235–9.

Johnson, C. G., Taylor, L. R. & Haine, E. (1957). The analysis and reconstruction of diurnal flight curves in alienicolae of *Aphis fabae* Scop. *Ann. Appl. Biol.* (in the Press).

Müller, H. J. & Unger, K. (1951). Über die Ursachen der unterschiedlichen Resistenz von *Vicia faba* L. gegenüber der Bohnenblattlaus *Doralis fabae* Scop. 1. Der Verlauf des Massenwechsels von *Doralis fabae* Scop. in Abhängigkeit vom Witterungsverlauf 1949 in Quedlinburg. *Züchter*, **21**, 1–30.

Müller, H. J. & Unger, K. (1952). Über den Einfluss von Licht, Wind, Temperatur und Luftfeuchtigkeit auf den Befallsflug der Aphiden *Doralis fabae* Scop. und *Myzodes persicae* Sulz. sowie der Psyllide *Trioza nigricornis* Frst. *Züchter*, **22**, 206–28.

Pearson, E. A. & Hartley, H. O. (1954). *Biometrika Tables for Statisticians*, vol. **1**, 238 pp. Cambridge University Press.

Taylor, L. R. (1951). An improved suction trap for airborne insects. *Ann. Appl. Biol.* **38**, 582–91.

Taylor, L. R. (1957). Temperature relations of teneral development and behaviour in alate *Aphis fabae* Scop. *J. Exp. Biol.* **34**, 189–208.

SPECTRAL SENSITIVITY OF CHROMATOPHORES IN *DIADEMA SETOSUM* (LESKE)*

By MASAO YOSHIDA†

Misaki Marine Biological Station, Misaki, Kanagawa-ken, Japan

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Classical accounts of the effect of coloured light on retinal pigment migration in frogs and crayfish have been reviewed by Parker (1932). Most of those results agree in indicating violet or blue as the most effective light. Zetter (1956) has shown that the light of wavelengths near to 540 mμ is most effective for the expansion of melanophores in frog's skin. On the other hand, the direct effect of light upon the iris, which responds like melanophores, shows that 486 mμ is the most effective wavelength (Weale, 1956).

In echinoids nothing is known about the relative effectiveness of various spectral regions in producing photic reactions, other than the findings of Millott & Yoshida (1956) in *Psammechinus miliaris*, where the blue-green region appears most effective. Recently, I have shown that chromatophores distributed over the epidermis of *Diadema setosum* respond directly to light (Yoshida, 1956). The present work aims at discovering the relative sensitivity of the chromatophores to light of differing wavelengths.

MATERIAL AND METHODS

The aboral region of the interambulacra of fresh specimens of *Diadema setosum* (Leske) was used in the present experiments, since the colour change was most evident here. Cut pieces of test were examined in running sea water at $25 \pm 1^\circ$ C. in darkness. Observations were not recorded until the pieces had been at least 1 hr. in darkness so as to be sure that the pigment in the chromatophores had concentrated fully. If any chromatophores still appeared stellate, such pieces were discarded.

Chromatophores were then subjected alternately to periods of 20 min. darkness and 15 min. light in the form of a spot projected on to the chromatophores as previously described (Yoshida, 1956). At the end of illumination, a quick observation was made and the stimulated chromatophore photographed. The results of observations as well as the photographs were used to determine the changes in the pigmentary condition of the cell.

The light for stimulating the chromatophores was obtained from a 6 V. 30 W. tungsten filament lamp, the brightness of which was controlled by the voltage applied. A voltage stabilizer was inserted in the circuit of the lamp. The lamp used was calibrated by Dr M. Okamatsu of the Electrotechnical Laboratory, Tokyo, so

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† Present address is Zoology Department, Bedford College, University of London.

that the colour temperature of the filament was known at 0·4 V. intervals between 2 and 5 V.

The filament was focused by a condenser lens on to the plane of the entrance slit of a double monochromator, the exit slit of which served as the source of monochromatic light for stimulating the chromatophores. The monochromator consisted of two SF 2 prisms of Perrin–Broca type, and the dispersion characteristics in the plane of the exit slit were calculated from the refractive index of the prisms at each wavelength and the focal length of collimator lenses. The width of each slit (entrance, central and exit) was fixed at 0·5 mm.

The stimulating apparatus was the same as that previously described (Yoshida, 1956). Here, however, the half mirror, from which the light beam was reflected so as to enter the optical system of the microscope, was made of Incomel metal evaporated on glass. Both the transmission and reflexion characteristics of this material were found to be fairly constant in the range 420–670 $m\mu$.

The minimum voltage necessary to make any perceptible change in pigment dispersion was measured for each wavelength, and the relative energy at such threshold voltage was obtained as follows.

The colour temperature at a given voltage was substituted in Wien's radiation formula, thus giving the spectral radiance of the filament. This value, divided by the value of spectral dispersion in the plane of the exit slit of the monochromator, gave the energy content of the light falling on the chromatophores, any slight spectral variation of the transmittance of light in the optical system being neglected. The energy was expressed in arbitrary units.

RESULTS AND DISCUSSION

As previously mentioned (Yoshida, 1956), difficulties arise when high intensities are used, because the pigment of the chromatophore may shift bodily with respect to the light source so that, after dispersion, the pigment does not necessarily return to its original position or conform to its original outline when concentrated. In experiments performed immediately after such a mass shift of the pigment had taken place, the minimal amount of light energy required to elicit the response was usually lower than before. In some experiments, the threshold values altered during the course of the experiment. The reason for this is unknown. Such experiments were considered unreliable and the results were not used.

The results shown in Table 1 and Fig. 1 were obtained from nine series of experiments, in which the threshold values for a given wavelength (usually 500 $m\mu$) were the same at the beginning and the end of each series. In order to make values in different series of experiments comparable, the results were recalculated so as to make the threshold unity at 500 $m\mu$. The second column in Table 1 shows the relative threshold energy which is necessary for the response. The reciprocal of the values expresses the relative sensitivity which is shown in the third column, the figures being adjusted to make the value 100 at 468 $m\mu$. Because of the superficial position of the chromatophores it is assumed that differential absorption in the

overlying regions of the skin is not likely to be significant. The chromatophores are sensitive over a broad band (450–500 mμ), and their maximal sensitivity is in the vicinity of 470 mμ.

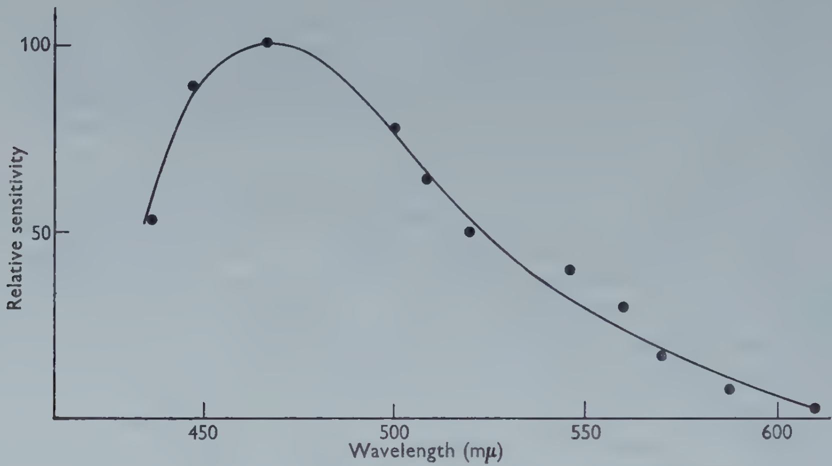

Fig. 1. Spectral sensitivity of chromatophores in *Diadema setosum.*

Table 1. *Spectral sensitivity of chromatophores in* Diadema setosum

Wavelength (mμ)	Relative threshold energy	Relative sensitivity
436	1·47	52·4
447	0·86	88·7
468	0·77	100
500	1·00	77·1
509	1·20	64·1
520	1·56	49·4
546	1·95	39·4
560	2·59	29·7
570	4·79	16·1
588	9·93	7·75
610	37·0	2·08
649	112	0·69

These results agree with the results obtained by Parker (1932), who showed that blue light was most effective in causing retinal pigment migration in frogs and crayfish, but not with Zetter's conclusions concerning the responsiveness of chromatophores in the frog's skin to coloured light. However, it is difficult to understand how Zetter reached his conclusions since the filters he used transmitted relatively broad bands (one from 460 to 600 mμ and the other from 600 to 690 mμ). It would appear that his results are not incompatible with a maximal sensitivity in the blue.

The region of the spectrum in which the chromatophores of *Diadema* will respond corresponds fairly closely with that in which the tube feet of *Psammechinus miliaris* react. It is thus possible that a common pigment may be involved.

There are also considerable differences between the spectral sensitivity of the chromatophores in *Diadema* and that of many complex photoreceptors. The cause of this must remain obscure until much more has been discovered concerning the photosensitive mechanism in *Diadema*.

SUMMARY

1. The spectral sensitivity of the chromatophores of *Diadema setosum* (Leske) was studied, using monochromatic light.

2. The chromatophores are most sensitive in the region of the visible spectrum between 450 and 500 mμ with a maximum at 470 mμ.

The author's thanks are due to the staff of the Misaki Marine Biological Station, to Prof. Z. Koana, Department of Physics, University of Tokyo, for various instructions in optics, to Dr M. Okamatsu for his kindness in calibrating the colour temperatures of the bulb, and also to Prof. N. Millott for his suggestions and help in preparing the manuscript.

REFERENCES

Millott, N. & Yoshida, M. (1956). Reactions to shading in the sea urchin, *Psammechinus miliaris* (Gmelin). *Nature, Lond.*, **178**, 1300.

Parker, G. H. (1932). The movement of the retinal pigment. *Ergebn. Biol.* **9**, 239–91.

Weale, R. A. (1956). Observations on the direct effect light on the irides of *Rana temporaria* and *Xenopus laevis*. *J. Physiol.* **132**, 257–66.

Yoshida, M. (1956). On the light response of the chromatophore of the sea-urchin, *Diadema setosum* (Leske). *J. Exp. Biol.* **33**, 119–23.

Zetter, A. (1956). Über die Lichtreaktion der Froschhautchromatophore. *Z. Biol.* **108**, 210–16.

ABSORPTION OF WATER BY THE EGG OF THE GARDEN CHAFER, *PHYLLOPERTHA HORTICOLA* L.

By ROGER LAUGHLIN*

University School of Agriculture, King's College, Newcastle upon Tyne

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Many insect eggs absorb water during the incubation period. Buxton (1932), Roonwal (1936) and Matthée (1951) review the numerous reports published since 1740 when Réaumur noticed that eggs of sawflies and ants increased in size during development.

A good deal of attention has been paid to the acridiid egg in which water enters through the hydropyle (Slifer, 1938), a specialized area of cuticle at the posterior end of the egg. The water content of the egg is closely correlated with the developmental stages of the embryo. However, the mechanism controlling water content is obscure and little understood. For example, Matthée (1951) shows that eggs of *Locustana pardalina* cannot absorb water in the absence of oxygen. He concludes that the hydropyle cells secrete water into the egg. Yet Slifer (1938) showed that the hydropyle cells of *Melanoplus differentialis* often become detached from the hydropyle at the beginning of blastokinesis without affecting the course of water uptake and embryonic development.

Among the lamellicorns, eggs of *Anisoplia austriaca* (Kerenski, 1930), *Melolontha melolontha* (Schuch, 1938) and *Popillia japonica* (Ludwig, 1932) have been studied. Water is absorbed over the whole shell and not through a specialized hydropyle.

Kerenski found that eggs of *Anisoplia* would swell and develop normally on filter-paper wetted with distilled water, soil filtrate, insect Ringer's solution, NaCl solution up to 4%, 2% KNO_3 solution or 2% $BaCl_2$ solution. Schuch notes that anything less than 100% relative humidity retards development, produces small larvae and increases egg mortality steeply. He also found that *Melolontha* eggs would develop normally if dipped in water (i.e. half submerged), but would die, sometimes swelling up and bursting, if completely submerged. Ludwig gives weight-increase curves for eggs of *Popillia* at two different temperatures (20 and 25° C.), showing that the rate of water absorption and the rate of development both increased at higher temperatures.

The garden chafer is a lamellicorn with an annual life cycle. In the field, adults are only seen for 4–6 weeks of the year (May–June). The females lay about a dozen eggs, depositing them singly about $1\frac{1}{2}$ in. deep, each in a small cavity in the soil. New-laid eggs are large and white (weight 1 mg., length 1·5 mm., diameter 1 mm.,

* Agricultural Research Council, Unit of Insect Physiology.

approximately) and their shape is usually that of a prolate spheroid. At least 95 % of eggs laid are fertilized and usually at least 90 % hatch. There is some evidence that the proportion of fertilized eggs decreases with the age of the female (Raw, 1951; Milne & Laughlin, 1956). In the field the embryonic period lasts a month or more, according to the prevailing soil temperatures.

Adults were kept at room temperature, twenty or thirty beetles to a cage, and provided with food and a tray of sifted soil. The females would burrow into the soil to lay and the eggs were sieved out once or twice a day. Thus the term 'new-laid eggs' refers to eggs less than 24 hr. old.

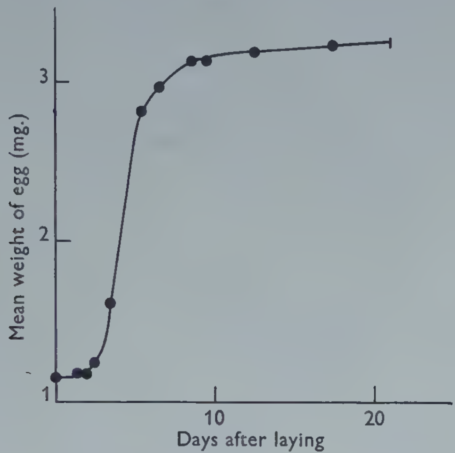

Fig. 1. Water absorption curve: the increase in mean weight of seven eggs, incubated at 20° C., with time.

ABSORPTION OF WATER

During the incubation period the egg trebles its weight by taking up water from the surroundings. The water content increases from 50 % in the new-laid egg to around 80 % in the swollen egg. The dry weight remains constant, as far as it is possible to tell from the following data: A batch of twenty eggs was divided into two lots of ten after the eggs had been weighed individually. One lot was oven-dried (12 hr. at 110° C.) and the eggs weighed again. The other lot was kept at 20° C. for 6–7 days in a damp atmosphere before drying. The mean dry weights of the two lots (0·574 and 0·569 mg. for new-laid and swollen eggs respectively) did not differ significantly ($P = > 0{\cdot}5$).

The water content of individual eggs of the same age varies very little. The water content of twenty new-laid eggs ranged from 48·4 to 51·9 % (mean 50·2 %) and of twenty swollen eggs from 79·6 to 83·1 % (mean 81·8 %).

At 20° C. the incubation period lasts about 21 days. For the first 2 days the egg remains chalky-white and sausage-shaped. On the second or third day the weight begins to rise and by the seventh day has doubled or trebled. The egg now appears pearly and translucent and is more nearly spherical in shape. From the seventh day to the end of the incubation period the weight continues to rise slowly (Fig. 1).

SHELL CHANGES DURING ABSORPTION

Twenty eggs were kept at 25° C. and weighed individually each day. Each day also, two or three eggs were taken, frozen into a drop of water by spraying drop and egg with ethyl chloride; the frozen egg was cut in half with a razor blade. The frozen half-eggs were immediately dropped into fixative and finally vacuum-embedded in paraffin wax for sectioning.

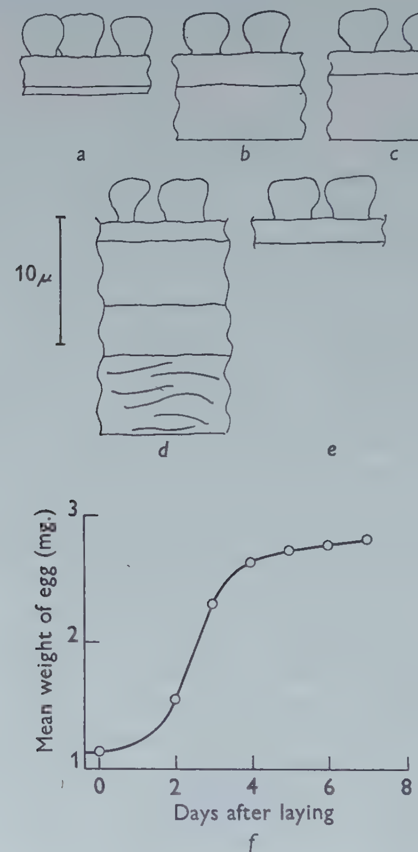

Fig. 2. Scale diagrams of shell sections (*a–e*) and water absorption curve of eggs at 25° C. (*f*). *a*, new-laid egg; *b*, *c* and *d*, swelling egg; *e*, vacated shell.

The chorion of the new-laid egg (Fig. 2*a*) is about 7 μ thick, the surface granules being 3–4 μ in diameter and the shell itself about 3·5 μ thick. Two layers can be seen at this stage, but only one layer of chorion is visible in older eggs. At 2–3 days (Fig. 2*b*) the serosa is present and the epembryonic cuticle already about 4 μ thick. At 3–4 days (Fig. 2*c*) the epembryonic cuticle is 4–5 μ thick and the serosal nuclei are darker and the cell boundaries less clearly defined. At 4–5 days the rate of water uptake is slowing down (Fig. 2*f*) and a second layer of epembryonic cuticle has been laid down (about 4 μ thick). There is also an inner layer (5–10 μ thick) which

appears in the sections as loosely packed fibres. The shell is now 9–11 μ thick, not counting the granules or the inner fibrous layer. It remains in this form until the embryo is ready to hatch. The epembryonic cuticle is then resorbed. Sections of vacated shell show only the chorion (Fig. 2*e*).

The chorion does not crack or break as the egg swells and so must stretch and become thinner. It measures 3–3·5 μ in the new-laid egg sections but only 1·5–2 μ in the swollen egg. In the vacated shell it is about 2 μ thick. The chorion is noticeably elastic. Strips of shell from a swollen egg always curl up with the granules on the inner side of the curve. Also, when the epembryonic cuticle is resorbed, the egg loses its shape and the chorion clings to the embryo. This plays an important part in the hatching mechanism, for the chorion is pressed closely against the egg-bursting spines on the third thoracic segment of the first instar larva (Rittershaus, 1925). At the least movement of the larva the spines pierce the chorion and the shell cracks and begins to slide off the emerging grub.

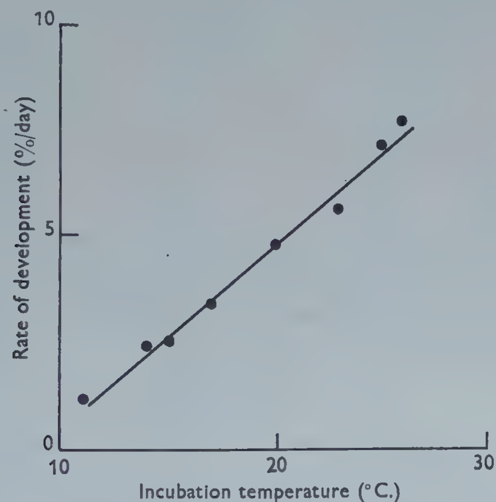

Fig. 3. The rate of development at different constant temperatures.

ABSORPTION AND TEMPERATURE

Eggs were kept in a damp atmosphere at various constant temperatures. The eggs were weighed individually at intervals to follow the course of water uptake. Absorption begins 1–6 days after laying (after 1 day at 26° C. and after 6 days at 11° C.). A period of rapid uptake follows, occupying about one-third of the total incubation period. The absorption rate then falls to a low level until the egg hatches.

Table 1 shows the length of the incubation period at various constant temperatures between 11 and 26° C. The rate of development (Fig. 3) appears to be proportional to the temperature. The rate of water uptake and the length of the rapid absorption period are also proportional to temperature. All three variables have the same temperature coefficient as far as it is possible to tell from Fig. 4. Fig. 4 shows

the mean water contents of three groups of eggs (incubated at 11, 17 and 25° C. respectively) plotted against time expressed as a percentage of the total incubation period.

Table 1. *Variation of rate of development with temperature*

Incubation temperature (°C.)	11	14	15	17	20	23	25	26
Length of incubation period (days)	86	42	40	29	21	18	14	13
Rate of development (percentage development per day)	1·2	2·4	2·5	3·4	4·8	5·6	7·1	7·7

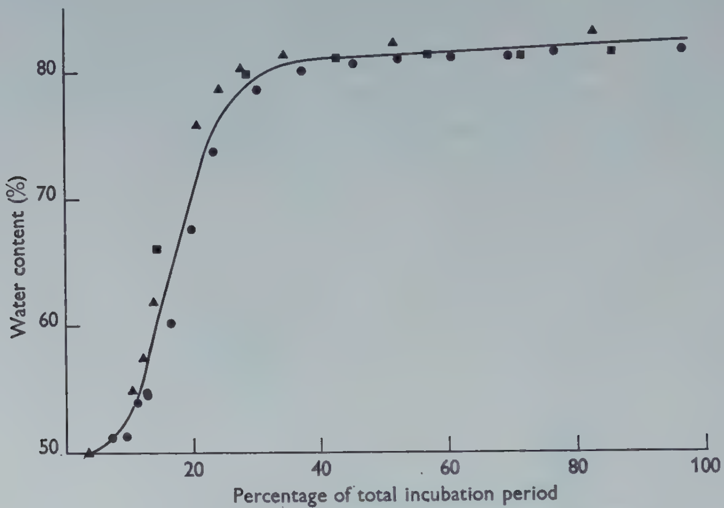

Fig. 4. Increase in water content with time expressed as percentage of total incubation period. ●, eggs at 11° C.; ▲, at 17° C.; ■, at 25° C.

DESICCATION RATE

The rate at which the egg loses water at 0% relative humidity changes with the age of the egg. To measure the desiccation rate a small torsion balance was made from a galvanometer by fixing a pan to the pointer. Eggs dropped in this pan deflected the pointer against the restoring spring of the instrument. The balance was calibrated and mounted in a square glass jar containing phosphorus pentoxide. The jar was then sealed. Eggs could be dropped into the pan through a tube without opening the jar. Normally the tube was also sealed. When the eggs had been desiccated they were ejected from the pan by discharging a condenser through the galvanometer to flick the pointer against a stop.

The sealed jar was kept at 20° C. and left for 4 days before taking the first reading. Eggs of different known ages were weighed and measured and dropped through the tube. The weight was read immediately and at intervals up to 5 hr. The successive weights were plotted against time, and from these curves the initial rate of water loss was calculated. This figure was divided by the surface area of the egg and the desiccation rate expressed as milligrams of water lost per square centimetre of surface per hour (Table 2).

The surface area of the egg was calculated from the length and breadth before desiccation, on the assumption that the shape was that of a prolate spheroid. It was also assumed that evaporation takes place over the whole surface of the egg. The shell appears to be quite uniform, in thickness and in surface appearance. No account was taken of the granular surface of the egg. The evaporating surface could be effectively higher or lower than the figures used, but any error should be similar for all eggs measured.

Table 2. *Rate of loss of water in dry air*

Age of egg (hr.)	Weight (mg.) before desiccation	Surface area (sq.cm.)	Desiccation rate (mg./hr.)
4±3	1·18	0·054	0·05
12±12	1·08	0·051	0·05
12±12	1·20	0·054	0·04
12±12	1·16	0·054	0·04
24±24	1·10	0·059	0·04
39±3	1·22	0·055	0·15
36±12	1·10	0·050	0·06
45±5	1·10	0·050	0·09
60±8	1·16	0·051	0·04
60±12	1·14	0·053	0·38
69±5	1·14	0·051	0·44
84±12	1·30	0·058	0·12
84±12	1·44	0·064	0·64
105±9	2·04	0·070	0·92
108±12	1·60	0·067	1·02
108±12	2·02	0·078	1·18
125±4	2·32	0·076	1·06
129±9	2·08	0·069	1·46
132±12	3·00	0·103	1·01
132±12	3·20	0·111	0·40
148±4	2·74	0·097	0·48
152±9	3·00	0·104	1·00
156±12	3·38	0·124	0·10
156±12	2·74	0·100	0·76
180±6	3·08	0·107	0·64
250±9	3·06	0·104	0·08
252±12	3·30	0·111	0·16

Fig. 5 shows the relation between the absorption curve (broken line) and the desiccation rate (continuous line) at 20° C. The desiccation rate curve is only intended to indicate the trend suggested by the data. On the first day after laying, the egg loses water at the rate of about 1 mg./sq.cm./hr. On the second day the rate increases, rising to a maximum of perhaps 20 mg./sq.cm./hr. at about the fifth day. Thereafter the rate falls and by the tenth day has dropped back to about 1 mg./sq.cm./hr. The desiccation rate starts to increase just before the egg begins to take up water and starts to decrease before the end of the rapid absorption period.

The rate of loss of water gives little information about the rate at which water can pass into the egg. It is well known that the surface membranes of insects show asymmetry, in that water passes across them more rapidly in one direction than the other (Beament, 1954). Furthermore, absorption of water may entail the condensation

of water vapour while desiccation requires the evaporation of water from the surface of the egg. However, changes in desiccation rate should reflect changes in permeability to inward flow.

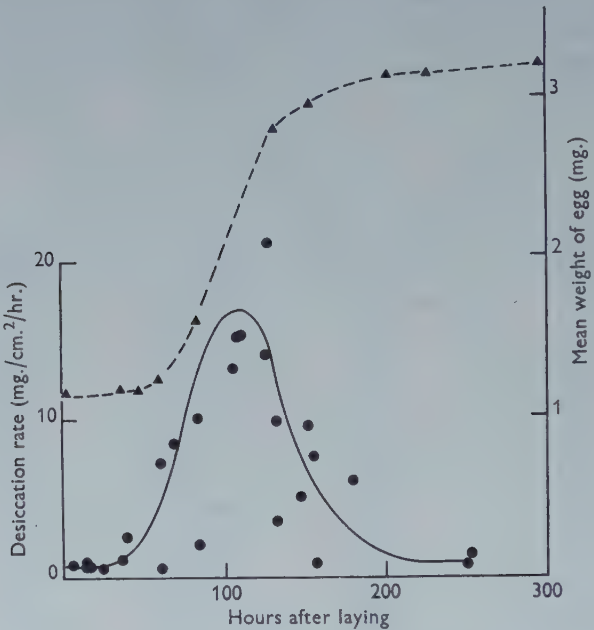

Fig. 5. Change in desiccation rate with age of eggs incubated at 20° C. ●, desiccation rate; ▲, absorption curve for eggs at 20° C.

OSMOTIC PRESSURE

Eggs were immersed in paraffin oil and pricked. A blob of fluid was squeezed out and a small sample drawn into a fine silica capillary. The capillary was sealed and its freezing-point determined as described by Ramsay (1949). Eggs were kept at 22° C. and the freezing-points of samples from eggs of different ages were determined. Eggs up to 4 days old were spun in an air centrifuge for $1\frac{1}{2}$–2 min. This separated out the fat droplets in the yolk which, if included in the sample, made the ice crystals very difficult to see. A centrifuged egg shows four layers. The fatty material forms a cap at one end with a clear region between it and the third layer. At the other end of the egg the densest constituents form a granular fourth layer merging into the third layer. Samples were taken from the third or fourth layers. By the fourth day after laying, at 22° C. the embryo has begun to form and there is an appreciable amount of extra-embryonic fluid. For eggs of this age and older, centrifuging was omitted and the sample drawn from the space above the embryo.

It was found that the freezing-point of a sample dropped rapidly at room temperature (about 20° C.). Thus one sample gave a reading of − 1·07° C. after 11 min. at room temperature and a reading of − 1·14° C. after a further 30 min. Two samples from another egg were frozen after 7 and 30 min. at room temperature respectively.

Their freezing-points were $-0{\cdot}92$ and $-0{\cdot}99°$ C. respectively. The drop is almost certainly due to autolysis of the organic constituents of the egg. The freezing-point (and hence the osmotic pressure) at the time of pricking must remain unknown, but it is evident that sampling must be done quickly and in a reasonably standard time. Samples were frozen within 12 min. of starting the centrifugc (average, 8 min.). Uncentrifuged eggs were sampled and the samples frozen within 3 min. of pricking. This difference in timing, and perhaps the centrifuging itself, affected the readings for centrifuged eggs. Three new-laid eggs which were sampled without centrifuging (i.e. frozen within 3 min. of pricking) gave significantly higher readings than the centrifuged new-laid egg samples (Table 3).

Table 3. *Osmotic pressure of yolk and extra-embryonic fluid*

Age of eggs (days)	No. of eggs sampled	Centrifuged (o) or not centrifuged (×)	Osmotic pressure (in atmospheres)	
			Mean	Range
0–1	6	o	12·7	11·9–13·9
0–1	3	×	11·5	11·3–11·7
1–2	10	o	12·8	9·3–17·0
2–4	6	o	13·2	12·6–15·0
4–6	7	×	10·3	8·7–11·8
7±8 hr.	5	×	9·2	8·9– 9·7
8±9 hr.	4	×	8·2	7·4– 9·3
10±7 hr.	4	×	7·3	7·3– 7·4

Thus there is an error in all the osmotic pressure values obtained. By standardizing the procedure the error is made, as nearly as possible, the same for all eggs sampled. In general the values given are too high, particularly in the case of eggs under 4 days old.

Table 3 shows the results obtained. The osmotic pressure was calculated from the relation

$$P = RT\frac{\Delta}{1{\cdot}86},$$

where P = osmotic pressure in atmospheres, Δ = freezing-point depression in ° C., T = absolute temperature, R = gas constant. The new-laid egg has an osmotic pressure of about 13 atm., which drops during the rapid absorption period to about 8 atm. in the swollen egg. Since the swollen egg contains about five times the quantity of water in the new-laid egg it is obvious that the embryo is controlling the concentration of soluble material in the yolk and extra embryonic fluid, perhaps by the breakdown of large molecules or by the 'binding' or utilization of incoming water.

ABSORPTION AND OSMOTIC PRESSURE

Eggs will sit on the surface of distilled water, absorbing water and developing normally. A series of solutions of sucrose was made up, giving osmotic pressures from 2·5 to 25 atm., with intervals of 2·5 atm. Fifty-five eggs between 2 and 3 days old at 20° C. (i.e. eggs which had just begun to swell) were divided into eleven groups

of five. Ten of the groups were floated on the ten sucrose solutions, and the eggs of the last group were weighed individually and floated on distilled water. All the groups were kept at 20° C. Five days later the eggs were washed in distilled water, rolled on filter-paper to dry off excess water, and weighed individually. Table 4 shows the figures obtained. The 'change-in-weight' line was obtained by subtracting the mean new-laid weight of the five eggs in distilled water from the mean weight of each group at the end of the experiment.

Table 4. *The water absorbed by eggs at different osmotic pressures*

Osmotic pressure of solutions (atm.)	Mean change in weight of 5 eggs in 5 days (mg.)
0	+1·96
2·5	+1·47
5·0	+1·74
7·5	+1·24
10·0	+0·68
12·5	+0·42
15·0	+0·11
17·5	0·00
20·0	+0·02
22·5	−0·16
25·0	−0·23

The amount of water absorbed by the eggs in 5 days decreases with increasing sucrose concentration. Above 15 atm. little or none is taken up. Above 15 atm. it would seem that the difference between the osmotic pressure of the solution and the force drawing water into the egg is not large enough to stretch the shell.

ABSORPTION AND HUMIDITY

New-laid and swollen eggs were suspended over distilled water and over saturated solutions of potassium bichromate or potassium nitrate at 25° C. These should have given relative humidities of 100, 98·00 and 92·48 % respectively (Solomon, 1951), but local fluctuations of temperature within the containers probably caused a good deal of variation from these figures.

The airtight jars containing solutions and eggs were looked at every 3 days but were not opened. At 92 % relative humidity all eggs, swollen and new-laid, collapsed within the first 3 days. At 98 % relative humidity the swollen eggs collapsed eventually but took rather longer to do so. The new-laid eggs managed to take up a little water at this humidity but not enough to allow the embryo to survive. In 'saturated air' the new-laid eggs absorbed water normally and all the eggs eventually hatched. Thus the new-laid egg cannot extract water from air that is much below saturation, and even swollen eggs are killed by prolonged exposure to unsaturated air.

DISCUSSION

The egg is not resistant to desiccation. Prolonged exposure to a relative humidity of 98 % or less kills both swollen and new-laid eggs. This is probably of very little importance in the field. The humidity of small cavities in soil under permanent pasture must rarely, if ever, drop below saturation.

In a saturated atmosphere water will tend to flow into the egg. To regulate absorption, water must be kept out. The process of regulation is highly efficient. The water contents of individual eggs of the same age and at the same temperature show very little variation; further, while rate of embryonic development varies widely with temperature, the water content of the egg remains closely associated with the stages of embryonic development.

The changes in desiccation rates suggest that the shell is relatively impermeable to water before and after the rapid absorption period; that the embryo removes a waterproofing layer, lets in so much water, and then re-erects some 'water barrier'. This may be partly true but is certainly not the whole explanation.

It seems quite likely that the new-laid egg depends chiefly on a waterproof shell to keep water out; unfertilized eggs usually remain small and unswollen for days until the whole system breaks down and decomposes.

The increase in shell permeability during the rapid absorption period facilitates the entry of water but does not necessarily control it. The rate of uptake during this period will also depend on the elasticity and plasticity of the shell. Yet neither of these physical mechanisms can explain the close relation between rate of uptake and embryonic development over a wide range of temperatures.

Similarly, low shell permeability and high hydrostatic pressure within the egg will tend to prevent entry of water in the latter part of the embryonic period but could not of themselves control the water content accurately.

SUMMARY

1. The garden chafer egg absorbs nearly twice its own weight of water in the early stages of the incubation period.

2. The chorion is thin, uniform and elastic, and the surface is covered with granules. Several layers of epembryonic cuticle are added as the embryo develops. These layers are resorbed before hatching takes place.

3. The rate of development, the rate of water uptake and the length of the rapid absorption period are equally affected by temperature.

4. Eggs are most easily desiccated in the rapid absorption period.

5. The yolk of the new-laid egg has an osmotic pressure of about 13 atm. The extra-embryonic fluid of the swollen egg has an osmotic pressure of about 8 atm.

6. Eggs absorb little or no water from sucrose solutions with osmotic pressures of 15 atm. or over.

7. New-laid eggs cannot absorb water from air at 98 % relative humidity. Swollen eggs placed in air of this humidity collapse and die.

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REFERENCES

BEAMENT, J. W. L. (1954). Water transport in insects. *Symp. Soc. Exp. Biol.* **8**, 94.

BUXTON, P. A. (1932). Insects and Humidity. *Biol. Rev.* **7**, 275.

KERENSKI, J. (1930). Beobachtungen uber die Entwicklung der Eier von *Anisoplia austriaca* Reitt. *Z. angew. Ent.* **16**, 178.

LUDWIG, D. (1932). The effect of temperature on the growth curves of the Japanese beetle (*Popillia japonica* Newman). *Physiol. Zoöl.* **5**, 431.

MATTHÉE, J. J. (1951). The structure and physiology of the egg of *Locustana pardalina* Walk. *Sci. Bull. Dep. Agric. S. Afr.* no. 316, pp. 1–83.

MILNE, A. & LAUGHLIN, R. (1956). Biology and ecology of the garden chafer, *Phyllopertha horticola* L. I. The adult and egg production. *Bull. Ent. Res.* **47**, 7.

RAMSAY, J. A. (1949). A new method of freezing-point determination for small quantities. *J. Exp. Biol.* **26**, 57–64.

RAW, F. (1951). The ecology of the garden chafer, *Phyllopertha horticola* L., with practical observations on control measures. *Bull. Ent. Res.* **42**, 605.

RITTERSHAUS, K. (1925). Eine neue Art von Eisprengern bei Lamellicornien-larven (*Phyllopertha horticola* L. und *Anomala aenea* DeG.). *Zool. Anz.* **62**, 31.

ROONWAL, M. L. (1936). Growth changes and structure of the eggs of the African migratory locust *Locusta migratoria migratorioides*. *Bull. Ent. Res.* **27**, 1–14.

SCHUCH, K. (1938). Ueber den Einfluss der Feuchtigkeit auf das Eistadium des Maikäfers (*Melolontha melolontha* L.). *Arb. physiol. angew. Ent.* **5**, 220.

SLIFER, E. H. (1938). Formation and structure of a special water absorbing area in the membranes covering the grasshopper egg. *Quart. J. Micr. Sci.* **80**, 437–547.

SOLOMON, M. E. (1951). Control of humidity with potassium hydroxide, sulphuric acid, or other solutions. *Bull. Ent. Res.* **42**, 543.

OSMOTIC CONDITIONS DURING THE EMBRYONIC AND EARLY LARVAL LIFE OF THE BROOK LAMPREY (*LAMPETRA PLANERI*)

By M. W. HARDISTY

College of Technology, Bristol

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I. INTRODUCTION AND METHODS

A considerable body of information has been accumulated on water exchanges and osmotic conditions in the eggs and early developmental stages of Amphibia and teleosts, but apart from some experiments by Bataillon (1909) and Yamamoto (1952) on the effects of saline solutions on early development, no studies of this type appear to have been made on the eggs and embryos of lampreys. The observations presented in this paper are intended as a contribution to the comparative study of these aspects of developmental physiology.

The ovarian egg of *Lampetra planeri* is distinctly ellipsoidal, measuring about $1{\cdot}06 \times 0{\cdot}94$ mm. along its longer and shorter axes. There is, however, considerable variation in the size of eggs taken from different females. Surrounding the ovum is a double-layered membrane about 0·04 mm. in thickness. The tough inner layer is referred to as the chorion, and this is covered by a gelatinous zone, initially of about the same thickness (Fig. 1*a*). The density of the egg is sufficiently great to allow it to sink quite rapidly in fresh water, when it immediately becomes adhesive and attaches itself firmly to any surface with which it may come into contact. The whole egg swells rapidly in the first few hours, and a fluid-filled space is formed between the chorion and the ovum (Fig. 1*b*). This is referred to as the perivitelline space. Accompanying the swelling of the egg there is a rapid expansion of the gelatinous outer coat, and as it expands the outline of this zone becomes less well defined and its optical density decreases. During this initial swelling the egg becomes more nearly spherical, its adhesiveness is lost and the density decreases with the uptake of water. These changes are normally so rapid that an egg shed into tap water loses its capacity for fertilization within an hour, although in 80–120 mM/l. Ringer made up according to the formula of Galloway (1933), swelling, expansion of the gelatinous layer and loss of adhesiveness do not occur. Eggs kept in such solutions may be fertilized successfully after a delay of up to 2 days. All the eggs used in this work were expressed from females caught in Somerset streams at the height of the spawning season in April and, except where expressly stated to the contrary, such eggs were immediately fertilized artificially in tap water.

For observations on the swelling of the egg the diameters along both axes were

measured with an eyepiece micrometer, using the same group of twelve eggs throughout the period of observation. Volumes have been calculated as ellipsoids from $V = \frac{1}{6}(\pi d_1 d_2)$, where d_1 and d_2 are the longer and shorter diameters. Prior to the first cleavage division, and also during those later stages when the blastula and gastrula are approximately spherical, the diameter of the ovum itself was measured, as well as the diameter of the whole egg. In the text and in Fig. 2, where reference is made to the volume of the whole egg, this does not include the gelatinous outer layer, which especially in the later stages of development becomes very irregular.

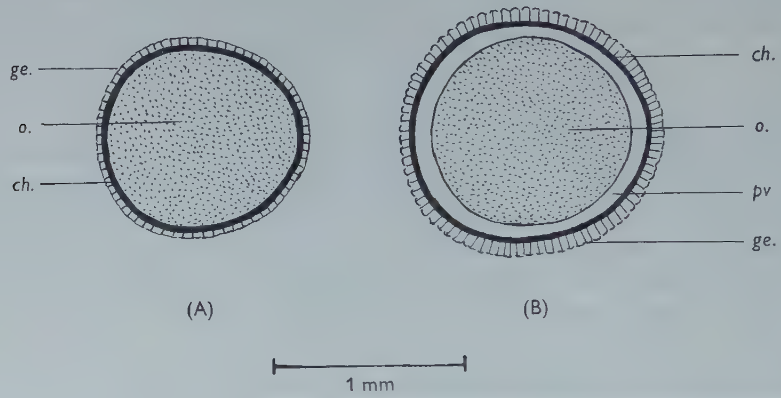

Fig. 1. A, ovarian egg. B, fertilized egg just before the first cleavage division at 8 hr. *o.* ovum; *ch.* chorion; *pv.* perivitelline space; *ge.* gelatinous outer membrane.

In determining the weight of the egg, batches of fifty eggs were pipetted in a single layer on to small tared circles of fine copper gauze. These were pressed on to a pad of filter-paper, sucked dry on a filter pump for 2 min. and then reweighed. This apparently crude procedure has been found in practice to give consistent results agreeing quite closely with other data. The dry weight was found by weighing the eggs on the gauze circles after drying in an oven at 105° C. for 24 hr. In the later stages of development it was possible to remove embryos from the egg membranes and weigh them in the same way.

The fluid used for vapour-pressure determinations was obtained by centrifuging whole eggs which had previously been subjected to the technique described above for the removal of surface water. Older embryos and larvae were crushed with a glass rod before centrifuging. Osmolar concentration was determined by the thermo-electric method using a Baldes thermocouple, and all measurements were made within half an hour of centrifuging. The results of the vapour-pressure determinations are expressed in terms of the corresponding NaCl solutions in mM/l. In the case of Cl determinations differential centrifuging has been used to separate the perivitelline fluid from the embryo. The eggs, supported in a tapered centrifuge tube by a small sieve constructed from a brass ring and fine copper gauze, were first centrifuged at about 900 r.p.m., which was sufficient to rupture the outer membranes and liberate the perivitelline fluid. Any contamination by

the egg contents is made immediately apparent by the presence of yolk in the otherwise quite colourless perivitelline fluid. The embryo fluid was subsequently obtained by centrifuging the egg at over 4000 r.p.m. to produce a clear yolk-free fluid. Duplicate determinations of Cl concentration were also made on the contents of whole eggs. The methods used for determining Cl concentration were similar to those described in a previous paper (Hardisty, 1956).

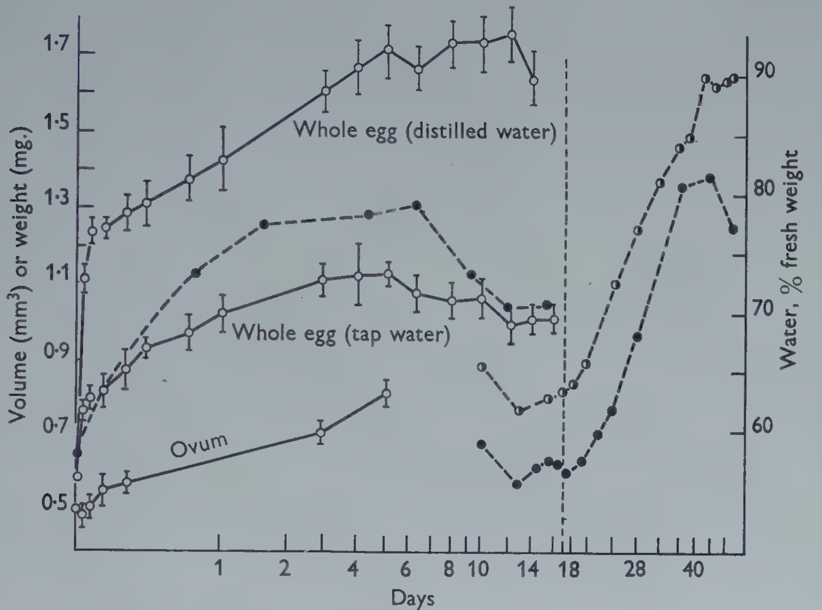

Fig. 2. Volume, weight and water content of ovum, whole egg, embryo and early larva. —○——○, volumes. Standard errors are indicated by vertical lines: -● - - ● - -, weight; - - ◑- - ◑ - -, water content percentage fresh wt. The vertical dotted line indicates hatching.

The total Cl content of the egg has also been found using dried material. About fifty eggs at a time were counted out from a pipette on to a weighed circle of oven-dried filter-paper 1 cm. in diameter. After 24 hr. drying at 105° C. the disks and eggs were re-weighed. The dried material was subsequently crushed with a glass rod on the filter-paper and transferred to a 2 ml. hard glass ampoule. After adding a measured volume of distilled water, the ampoule was sealed off and digested in an oven at 100° C. for 24 hr. The inverted ampoule was then centrifuged to remove the paper and egg residue and aliquot portions of the fluid were drawn off from the stem of the ampoule. Cl analyses were carried out on this fluid by the methods to which reference has been made. Quantities of Cl have been expressed as μM Cl/100 eggs or in some cases as mM Cl/l. of egg water.

As this work has, of necessity, been carried out over several years and as it has not been possible to rear the fertilized eggs at exactly the same temperature each year, there have been some differences in the rate of development and the time of hatching. Thus, in 1953 at 11–12° C., hatching began after 17 days and in 1956,

at 9–10° C., after 21 days. In distilled water hatching usually appeared to occur a day or two earlier than in tap water, while saline solutions seem to have a retarding effect on development. Eggs have been successfully reared in a refrigerator at temperatures of 3–5° C. when hatching began about 8 weeks after fertilization. When there have been pronounced differences in the duration of the embryonic period the time scale has been standardized in Fig. 3 by reducing each group to a pre-hatching period of 17 days.

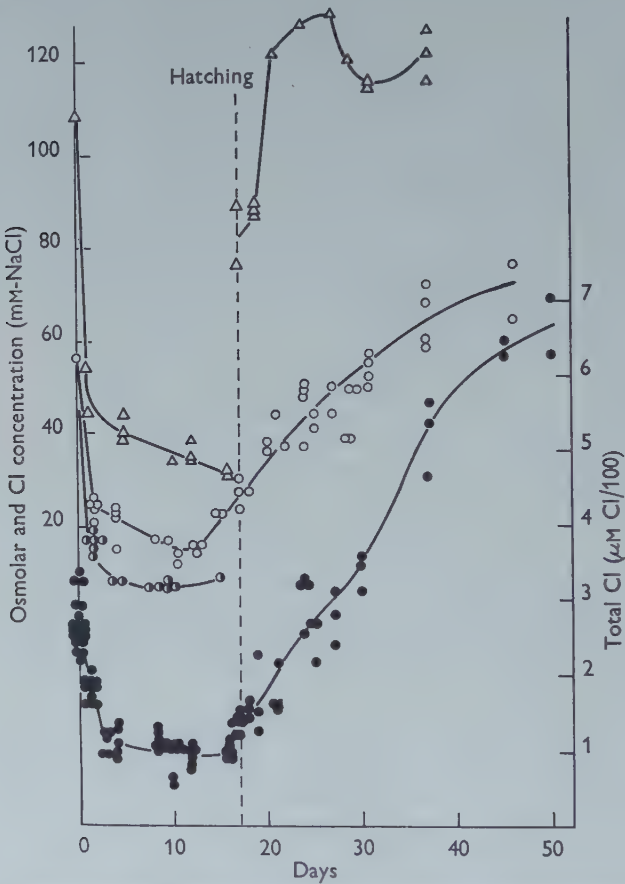

Fig. 3. Osmolar concentration, Cl concentration and total Cl content during embryonic and early larval development. –△——△, osmolar concentration of whole eggs and larvae (mM NaCl/l.); –○——○, Cl concentration of egg, embryo and larva (mM Cl/l. embryo or larval fluid); –◑——◑, Cl concentration of whole egg (mM Cl/l. egg fluid); –●——●, total Cl content of egg, embryo or larva (μM Cl/100 eggs). Hatching is indicated by the vertical dotted line.

II. CHANGES IN WEIGHT, VOLUME AND WATER CONTENT

In one of a series of observations on the changes in diameter of the fertilized egg measurements were extended over the whole of the embryonic period, both in distilled and tap water (Fig. 2). After 45 min. the total volume of the egg had increased in tap water by about 30% and in distilled water by over 90% on the

initial volume. In both groups the rate of swelling fell off sharply after a few hours, although continuing at a much reduced rate up to the fifth day, when the volume in tap water had increased by over 90 %. In distilled water the volume of the whole egg was doubled in the first 2 hr. and by the fifth day was more than three times the initial volume. In both tap and distilled water there appears to be an inflexion in the volume curve after 5 days, and in tap water the volume actually decreased from this point up to hatching.

The formation of the perivitelline space was to a great extent accomplished during the first 45 min. Thus at the end of this time the volume of the perivitelline fluid, for the egg in tap water, had already reached 70 % of the maximum volume recorded on the third day. In distilled water this maximum volume was 0·93 $mm.^3$ compared with 0·40 $mm.^3$ in tap water. It should, however, be noted that this increased volume in distilled water is due mainly to greater swelling in the first 45 min., and at the end of that time the perivitelline volume was already double that of the egg in tap water.

The swelling of the outer gelatinous layer follows a very similar course to that of the egg as a whole, reaching 70 % of its maximum depth within 45 min. of contact with water, and no doubt this swelling is responsible for the loss of adhesiveness at this time. This zone reaches its greatest development within 3 days, and a few days later shows signs of deterioration, finally sloughing off during the last week of embryonic life leaving the chorion exposed. In distilled water this layer was eventually about twice as thick as in tap water, but at any rate in the early stages it is much less dense and so attenuated that its outline was hard to define. In 80 and 120 mM/l. Ringer solutions on the other hand, it usually failed to swell or if it did so the swelling was only slight. These circumstances have much in common with the swelling of the mucus of the frog's egg in which the expansion in distilled water is much more pronounced than in tap water or low concentrations of single salts (Krogh, Schmidt-Nielsen & Zeuthen, 1938).

Considering the ovum itself, no swelling was detected in the first 45 min., and indeed in tap water a slight decrease in the mean volume was recorded at the end of this period. Although the difference is statistically hardly significant, a similar decrease has been observed in other measurements, and it is quite possible that there is a real contraction similar to that claimed by Bialaszewicz (1908) for the egg of the frog. During the next hour the swelling of the ovum was pronounced and amounted to an increase of about 8 %, but in the following 6 hr. declined to about 1 % per hour. At no stage was there any significant difference in the swelling of the ovum in tap and distilled water.

To assist comparison of water transport through the external membranes and the surface of the ovum the rate of uptake of water has been calculated over various time intervals both for the ovum and the whole egg (Table 1). These figures show a sharp reduction in the rate of uptake after the first 2 hr. through both the surface of the ovum and the outer egg membranes. For the ovum the rate of uptake continued to decrease up to 7 hr., but from this time until the fifth day remained constant. While initially the rate of transport through the outer membranes was

very much greater than through the surface of the ovum, the difference becomes less pronounced as development proceeds. Indeed, as previously pointed out, the perivitelline volume is not significantly altered in the period 0·75–3·75 hr. Similarly, although initially the rate of uptake through the outer membranes in distilled water was over twice as great as in tap water, the difference in the two media becomes subsequently much less important.

Table 1. *Calculated rates of uptake of water by the whole egg and the ovum alone for certain time intervals*

Period (hr.)	Rate of uptake of water (μ^3/μ^2/min.)		
	Whole egg		Ovum
	Distilled water	Tap water	Tap water
0 –0·75	2·660	1·040	—
0·75–1·75	0·301	0·269	0·230
1·75–3·75	0·054	0·040	0·043
3·75–7·00	0·020	0·056	0·027
7–80	0·013	0·011	0·009
80–128	0·006	0·0002	0·009

Owing to the presence of the outer gelatinous layer, the weights recorded for the whole egg are not strictly comparable with the volume data used in the preceding discussion, since the latter are based on the diameter of the egg measured to the outer surface of the chorion. For this reason, presumably, the increases in weight recorded during the initial swelling of the egg are rather greater than the increases in volume at this time. Thus, for the same eggs as were used for volume measurements, the maximum weight was recorded on the fifth day (Fig. 2) when the fresh weight had increased by about 90 % compared with an increase in volume of 84 % during the same period. The decrease in the fresh weight of the whole egg which occurred during the later stages of embryonic life is due, in part at least, to the shedding of the gelatinous coat, but the coincidence of the decrease in both volume and fresh weight suggests that a contraction of the chorion does in fact occur and that this may well contribute to the disintegration of the outer layer in the last few days before hatching.

Comparison of the measured volumes with the weights of decapsulated embryos either on the point of hatching or several days before, reveals marked discrepancies between the 5-day gastrula and the embryo shortly before hatching (Table 2). For the unfertilized egg the figures in the table are not strictly comparable, since the volume is that of the ovum alone while the weight includes the outer membranes. If the density of these membranes is assumed to be similar to that of the egg as a whole (1·05), the weight of the ovum alone may be estimated as 0·534 mg. The observations made on volume changes indicate that during the first 5 days of development 0·31 mm.^{3} of water is absorbed by the embryo, so that the fresh

weight of the gastrula at this stage could hardly be less than 0·84 mg. This estimate, of course, ignores the changes in dry weight which decreases by about 20 % of its initial value during the whole embryonic period. Over the first 5 days therefore, the decrease in dry weight would be no more than about 0·02 mg. The large number of observations on embryos between 11 days and hatching appear to indicate a reduction in the fresh weight of about 0·2 mg. or about 24 % as compared with

Table 2. *Volume and fresh weight of embryos at various periods of development*

Stage of development	Mean volume (mm.^{3}, S.E.M.)	Mean fresh weight (mg., S.E.M.)
Unfertilized egg	0·509 ± 0·007	0·633 ± 0·017
3-day blastula	0·709 ± 0·007	—
5-day gastrula	0·816 ± 0·008	—
11–13 days	—	0·612 ± 0·019
15–16 days	—	0·618 ± 0·035
17 days (hatching)	—	0·617 ± 0·012

5-day gastrula. As a result, the water content of the embryo at hatching was found to be only slightly greater than that of the ovarian egg. Taking the water content of the unfertilized ovum as 0·31 mg. or 57 % of the fresh weight, this would be increased in the gastrula stage to 0·62 mg. or 73 % of the estimated fresh weight at this stage. However, the mean water content for all decapsulated embryos during the period 11–16 days was only 0·38 mg. or 62·5 % of the fresh weight. Although this point merits further investigation, it is difficult to resist the conclusion that there is a considerable reduction in both the volume and weight of the embryo at (or shortly after) gastrulation, coinciding with the contraction observed in the outer membranes. In this connexion it may be noted that the temporary increase in density observed during gastrulation in amphibian embryos (Dempster, 1933; Briggs, 1939) has been related by Brown (1941) to the collapse of the archenteron roof and the consequent loss of fluid through the open blastopore. However, in amphibian embryos the decrease in volume of the neurula is almost immediately followed by a further pronounced and continuous increase in volume which lasts for the remainder of embryonic life. Examination of the recorded weights of the decapsulated lamprey embryos between the eleventh day and hatching fails to reveal any significant trend, and if further uptake of water occurs it must be on a very reduced scale compared with the rates observed prior to gastrulation. Cambar (1947*a*, *b*) has shown that in the frog embryo the pronephros at hatching excretes as much as 30 % of the fresh weight of water per day. Moreover, it begins to function 4 days before hatching, and within a day or so its output increases rapidly. In the lamprey embryo the exact point at which the pronephros becomes functional has not been determined, but certainly it appears that the pronephric ducts are open at the time of hatching (Wheeler, 1899). The possibility of active regulation of water content in later embryonic development cannot therefore be entirely excluded.

The conclusions reached regarding the decrease in embryo volume receive some additional support from information on the volume of the perivitelline fluid at hatching. Thus, taking this volume as the difference in the weight of the hatched embryo and the whole egg just before hatching, the mean value was 0·41 $mm.^3$, whereas at 5 days the difference in the volume of the whole egg and embryo was only 0·34 $mm.^3$ In view of the reduction recorded in the volume of the whole egg between the fifth day and hatching, these figures must be taken as implying a considerable reduction in embryo volume within this period.

As is the case with amphibian larvae (Dempster, 1933; Cambar, 1947*b*) the fresh weight increases after hatching with the uptake of water, although, owing to the logarithmic time scale, Fig. 2 does not give a clear picture of the rate of increase in weight which is far from linear. For the first 5 days after hatching the fresh weight increased by about 14% on the weight of the embryo at hatching, but for the following 5 days the rate of increase was 35%. Within 2 weeks of hatching the larval fresh weight had almost doubled and the maximum weight was recorded 3–4 weeks after hatching. During the whole of this period of larval growth the water content of the ammocoete was raised from 63% of the fresh weight at hatching to over 90%. This latter figure is much higher than the values found for ammocoetes taken from the stream (78–84%, Hardisty, 1956); but where, after the absorption of the yolk, ammocoetes have been successfully fed, the water content was subsequently reduced to 85%.

In embryos and tadpoles of the frog it has been shown (Cambar, 1947*b*) that differences in the rate of water retention are due, not to variations in the output of the pronephric or mesonephric kidney, but rather to differences in the rate of water uptake via integument or gut. It seems likely that the increased rate of water uptake which appears in the ammocoete in the second week after hatching is associated with the perforation of the mouth and branchial pouches.

III. CHANGES IN THE OSMOLAR AND CHLORIDE CONCENTRATION

For unfertilized eggs from three animals duplicate vapour-pressure readings gave mean values of 80, 90 and 106 mM/l., but the lowest figure was for an animal which had been kept for over a week in the laboratory, and previous experience has shown that, where spawning is delayed, the body cavity tends to become distended with peritoneal fluid of abnormally low concentration. In a very large number of observations the osmolar concentration of the peritoneal fluid was found to lie between 80 and 130 mM/l. with a mean value of 110 mM/l. (Hardisty, 1956), of which nearly 90% is accounted for by Cl and its associated univalent cations. Observations on the effects of Ringer solutions on the diameter of the unfertilized egg showed that in 80 mM/l. solutions there was no significant change, but that 120 mM/l. caused slight shrinkage (Table 3). Nevertheless, in both these solutions the eggs retain their capacity for fertilization and subsequent development.

Bataillon (1900) reported that the eggs of *L. planeri* failed to segment in a 1% solution of NaCl (171 mM/l.) or in isosmotic solutions of cane sugar or $CaCl_2$,

Table 3. *The effects on the diameter of the unfertilized egg of* 80 mM/l. *and* 120 mM/l. *Ringer solutions. The diameters are the mean of the longer and shorter axes*

Time (hr.)	Ringer (80 mM/l.)		Ringer 120 mM/l.	
	Whole egg (mm. S.E.M.)	Ovum (mm. S.E.M.)	Whole egg (mm. S.E.M.)	Ovum (mm. S.E.M.)
0	1·06 ± 0·005	1·000 ± 0·003	1·05 ± 0·015	1·028 ± 0·013
4	1·047 ± 0·004	0·993 ± 0·004	1·00 ± 0·009	0·94 ± 0·007
11	1·055 ± 0·007	1·005 ± 0·004	0·98 ± 0·005	0·94 ± 0·005
	Significance of differences between means			
0–4 hr.	$P>0·5$	$P>0·5$	$P=0·01-0·001$	$P=0·001$
0–11 hr.	$P>0·8$	$P>0·7$	$P=0·001$	$P=0·001$

while in solutions corresponding to 0·8% NaCl (137 mM/l.) cleavage did not proceed beyond the 16-cell stage. In the unfertilized eggs of *L. reissneri*, Yamamoto (1952) observed neither shrinkage nor swelling in M/7 Ringer (143 mM/l.), although the eggs swelled slightly when returned from this solution to M/16–M/500 Ringer, presumably owing to the penetration of ions into the egg from the M/7 solution. In view of the permeability of the egg to water and ions, experiments of this type can hardly be expected to yield very precise information on the concentration of the egg fluid. In general, previous work on the eggs of Amphibia (Krogh, Schmidt-Nielsen & Zeuthen, 1938; Backman & Sundberg, 1912) or teleosts (Runnström, 1920) has shown close agreement in the concentrations of body fluids and of unfertilized eggs, although Picken & Rothschild (1948) obtained considerably higher vapour-pressure readings for the egg of the frog compared with blood. Taking into account all the available information it seems safe to assume that the ovarian egg of the lamprey is isosmotic with the peritoneal fluid.

For six batches of freshly expressed eggs the mean Cl concentration lay between 50 and 70 mM/l. of egg fluid with a mean value of 56 mM/l., which is similar to the Cl concentration of adult muscle extracts (Hardisty, 1956). Somewhat higher Cl levels are, however, implied by the determinations made on the total Cl content of the dry egg. Thus for twenty-one samples of unfertilized eggs the Cl content varied from 2·22 to 3·4 μM/100 eggs (mean 2·68), representing a Cl concentration in the total egg water of 71 mM/l. The differences between the two Cl values, 56 mM/l. egg fluid and 71 mM/l. egg water, would be reduced if account was taken of the water content of the egg fluid (obtained by centrifuging). This fluid presumably contains soluble colloids in considerable amounts. Another factor which may contribute to the high initial values for the total Cl content of the dry egg is the adsorption of Cl ions in the gelatinous outer envelope. In the mucus of the frog's egg Krogh *et al.* (1938) found that the Cl concentration rapidly reached equilibrium with the surrounding medium, which in the present case is the peritoneal fluid with a high Cl concentration. It may be estimated that, if this holds true for the egg of the lamprey, the Cl content of the outer membrane would be about 0·3 μM/100

eggs. This would reduce the Cl content of the ovum itself to about 2·4 μM/100 eggs, representing a Cl concentration of 65 mM/l. However this may be, it is clear that, while the egg may well be isosmotic with the peritoneal fluid, it is certainly not in ionic equilibrium with this fluid.

The rapid inflow of water into the perivitelline space (and to a lesser extent into the ovum itself) in the early stages is reflected in the sharp fall in both osmolar and Cl concentrations (Fig. 3). In Table 4 the observed values are compared with those calculated from $p_t = p_0 V_0 / V_t$, where p_0 is the initial osmolar or Cl concentration, V_0 the initial volume of egg water, V_t the volume of water after time t and p_t the osmolar or Cl concentration at that time. From the mean of numerous weighings the water content has been taken as 0·36 mm.^{3} and the values of V_t have been found by the addition of the appropriate volume increments. The initial osmolar concentration has been taken to be 110 mM/l. and the Cl concentration as 56 mM/l.

Table 4. *Comparison of observed and calculated values for the osmolar and* Cl *concentration of the egg*

Time (days)	Osmolar concentration (mM/l. NaCl) Whole egg		Cl concentration mM/l. egg or embryo centrifugate			
			Whole egg		Embryo	
	Observed	Calculated	Observed	Calculated	Observed	Calculated
0	110	—	56	—	—	—
1	49	52	17	26	24	54
5	40	44	8	22	21	32
10	34	46	7	24	—	—

For the first and fifth days agreement between observed and calculated values of osmolar concentration is very close, but the reduction in volume which is reflected in a somewhat higher calculated concentration on the tenth day is not borne out by the observed values. The observed Cl concentrations of whole eggs during the embryonic period are much lower than the calculated values, which is hardly surprising in view of the losses of Cl ions from the egg in the early stages, to which reference is made later in this section. However, it should be pointed out that the volume measurements on which the calculated values are based were carried out in 1953 when the vapour-pressure determinations were also made. The Cl concentrations, on the other hand, were obtained in 1955 when the average temperature of the water was some 2° C. lower than in 1953, and for these eggs increases in weight were considerably greater than those observed in the earlier year. This would, presumably, imply a greater perivitelline volume and lower concentrations for the whole egg. Certainly, a limited number of Cl determinations made in 1953 gave distinctly higher values compared with those obtained in 1955. However, the reliability of the values in Table 4 is confirmed by the excellent agreement shown with those derived from the total Cl content of the dry egg (Table 5). The same disparity between observed and calculated Cl concentrations is seen in those Cl

analyses made on the embryo separately. For six samples of embryos 36 hr. after fertilization Cl concentrations varied from 21 to 26 mM/l. embryo fluid (mean 24 mM/l.) and within the period 8–12 days, 11–18 mM/l. (mean 15 mM/l.). Three further determinations 2 days before hatching gave values of 17, 23 and 28 mM/l.

No separate measurements were made of the osmolar concentration of the embryo itself, but, if we assume that the ratio osmolar concentration of whole egg/osmolar concentration of embryo is similar to the ratio of their Cl concentrations, it may be estimated that, for the period 10–12 days, the total concentration of the embryo would be about 71 mM/l. Observed values for embryos either on the point of hatching or shortly afterwards were 76, 87, 89 and 90 mM/l. The available information appears to show that, although Cl levels are much lower than might be expected from the known dilution of the egg fluids, the osmolar concentration does not fall below the estimated levels. In view of the considerable losses of Cl ions in the first day or so, the conclusion seems inescapable that these losses are offset by the production of osmotically active metabolities which are retained within the embryo.

The low values found for the Cl concentration of the perivitelline fluid showed a tendency to decrease as development proceeded. For the first 4 days after fertilization the mean value was 7·4 mM/l.; for the eighth to twelfth days, 3·5 mM/l. and from the thirteenth to seventeenth days 2·4 mM/l. These low concentrations are in agreement with conditions in the eggs of Amphibia (Krogh *et al.* 1938; Richards, 1940) and teleosts (Svetlov, 1929). For the tap water used, the electrolyte content may be given as 7·0 mg. ions/l. or 3·5 mM/l., so that the perivitelline fluid, at any rate in the later stages of development, may well be in osmotic equilibrium with the surrounding water.

The validity of the technique employed in making separate Cl determinations on embryo and perivitelline fluid may be assessed by calculating the volume of fluid in the embryo from the Cl data and comparing this figure with that derived from volume and weight measurements. Following a procedure similar to that used by Svetlov (1929), the formula for the Cl concentration of the egg may be expressed by $V_e = \dfrac{V(k_p - k)}{k_p - k_e}$, where V is the total volume of the egg fluid, V_e the volume of fluid in the embryo and k, k_e and k_p the Cl concentrations of the whole egg, embryo and perivitelline fluid respectively. Applying this expression to the Cl concentration at two periods, 8–12 and 15–17 days, the estimated volumes work out as 0·40 and 0·35 mm.^{3} compared with volumes of 0·38 and 0·39 mm.^{3} from volume and weight data.

Shortly before hatching there were some indications of a rise in Cl concentration, and this increase continued throughout the whole period of larval life up to the time when the yolk was completely absorbed. During the first week after hatching the osmolar concentration rose rapidly from 85 to 120 mM/l. and the Cl concentration from 24 mM/l. 1 day before hatching, to 43 mM/l. In the second week the osmolar concentration showed little further increase and indeed, towards the end of this period, began to drop slightly. As already pointed out, the decrease in osmolar

concentration during the embryonic period (from 110 to 85 mM/l.) is no greater than the drop in Cl concentration alone (56–25 mM/l.). However, as a result of the rise in Cl concentration in the early larval period, the Cl fraction of the total concentration is once again raised until it reaches a value (50 %) similar to that encountered in the unfertilized egg and in the serum of older ammocoetes (Hardisty, 1956).

Analyses of the total Cl content of eggs and embryos demonstrate a marked loss of Cl ions in the first 2 days after fertilization (Table 5), during which time the Cl content is halved. While the presence of dead or unfertilized eggs might, of course, tend to reduce the apparent Cl content of the developing eggs, this source of error is not thought to be serious in view of the large number of eggs in each sample and the fact that there was little further reduction after the first 2 days. Earlier in this section reasons were given for believing that the initial value might be raised by a high Cl concentration in the outer gelatinous membrane, but this could not seriously affect the general result. Having regard to the extent of the Cl losses, it is rather surprising that the egg should develop in distilled water, at any rate up to hatching and usually for a week or so afterwards.

Table 5. *Mean* Cl *content of whole eggs at various periods in development*

State of development	Cl (μM/100 eggs	Cl concentration calculated as mM/l. egg water	Observed Cl concentration mM/l. egg fluid
ovarian egg	2·68	71	56
1 day	1·80	17	17
2 days	1·09	9	—
4 days	1·12	9	8
10 days	0·92	7	7
16–17 days (hatching)	1·39	35	24

The active uptake of Cl, which apparently begins shortly before hatching, is most marked in the second week, and this is probably associated with the opening of the mouth or branchial clefts. Within 35 days after hatching the Cl content was raised fourfold, i.e. from 1·39 to 6·5 μM/100 larvae and, taking into account the increasing water content, the overall Cl concentration would be increased from 35 to 51 mM/l. in the same period. Expressed in terms of dry weight, these Cl values are more than twice as great as those observed in ammocoetes taken from the stream and even higher than those of the adult brook lamprey at spawning time (Hardisty, 1956). Expressed as mM/kg. fresh tissue, however, the early larval Cl content is almost identical with that of the adult lamprey, although still considerably greater than that of normal ammocoetes. However, from what is known of the relationship between nutritional state and Cl levels in lampreys (Hardisty, 1956) these high Cl figures are hardly surprising.

IV. DISCUSSION

The significance of changes in water uptake in relation to the permeability of the egg surfaces can only be assessed if account is taken of the decrease in concentration resulting from dilution of the egg contents. Accordingly, the osmolar concentration for the ovum has been estimated from p_0V_0/V_t (see p. 246) for the various time intervals in Table 1, taking for p_0 a value of 110 mM/l. or 4·5 atm. Using these values, the permeability of the surface of the ovum for the first hour after swelling began is estimated as 0·049 μ^3/μ^2/atm./min., which is almost identical with the figure derived from the day number quoted by Krogh *et al.* (1938) for the egg of the frog in the initial period of 4 hr. (0·05 μ^3/μ^2/atm./min.). During the next period of 5 hr. the permeability drops sharply to 0·007 and thereafter remains constant throughout the first 5 days at 0·003.

In view of the rate of water uptake by the whole egg it is quite clear that the initial permeability of the outer membranes is altogether greater than that of the surface of the ovum, but the extent of possible changes in the permeability of these membranes is difficult to assess, since neither the initial volume nor the initial concentration of the perivitelline fluid is known. The flow of water into the perivitelline space is presumably due to the presence, below the chorion, of osmotically active substances, which may well be secreted by the ovum at the moment of contact with water (Bialaszewicz, 1908; McClendon, 1915). Bearing in mind the extent to which the perivitelline fluid must be diluted within the first hour, it is safe to assume that the absolute permeability of the outer membranes must be even greater than the rates of uptake indicate (Table 1). Further, the reduction in uptake which occurs after the first 2 hr. must reflect a real change in the permeability of these membranes, since during the period (1·75–3·75 hr.) the perivitelline volume was virtually unchanged. The initial changes which have been described are independent of fertilization, although this may well influence the rate of swelling. Such experiments as have been made with unfertilized eggs, although rather inconclusive, seem to point to a slightly lower rate of uptake as compared with the fertilized egg.

The difference in uptake in tap and distilled water affects only the perivitelline space, which in distilled water reaches a volume more than double that in tap water. According to Krogh *et al.* (1938) the egg of the frog often bursts in distilled water, and balanced solutions appear to be necessary for the reduction in permeability which occurs normally after a few hours in tap water. It is, however, by no means certain that reduced permeability need be invoked to explain the lower rate of uptake into the perivitelline space in tap water. Taking the concentration of the tap water as 3·5 mM/l., the osmotic gradient in this medium may be expressed as $\Delta - 3{\cdot}5$ mM/l. (where Δ is the concentration of the perivitelline fluid) and the concentration gradient in distilled water will be Δ. Then, making use of the rates of uptake for the first 45 min. (Table 1), we have: $\dfrac{\Delta}{\Delta - 3{\cdot}5} = \dfrac{2{\cdot}66}{1{\cdot}04}$, from which

$\Delta = 5{\cdot}8$ mM/l. Although this figure seems rather low, it cannot be entirely dismissed in view of the known Cl concentration of the perivitelline fluid and the extent of the dilution which occurs at this time. Neither should the possibility be overlooked that the greater depth of the gelatinous layer may exert some influence on the permeability of the outer membranes in the early stages. The gradual loss of this layer in the last week before hatching would not be expected to have much effect, since by that time the perivitelline fluid would be approximately in osmotic equilibrium with the water. It is not inconceivable that the attenuation and lack of density of the gelatinous layer in distilled water may contribute to the greater initial rate of uptake through the outer membranes in this medium.

In regard to water exchanges and permeability to electrolytes it seems that the egg of the lamprey resembles the amphibian rather than the teleost egg. The latter appears to develop, within a very short time of contact with fresh water, a high degree of impermeability to both electrolytes and water (Gray, 1932; Manery & Irving, 1935; Krogh & Ussing, 1937; Loeb & Wasteney, 1915), although there is evidence that water exchanges on a reduced scale may continue throughout development (Vernidub & Leyzorovitch, 1950). With the possible exception of the egg of *Hynobius retardatus*, where the concentration of the egg contents is maintained at a constant high level (Kusa, 1952), the amphibian embryo continues to absorb water throughout the whole embryonic period except for a short time during gastrulation. Amphibian development is therefore accompanied by a more or less marked reduction in concentration, although this may not be as great as Backman & Runnström (1912) believed to be the case in the frog's egg. For the amphibian egg the evidence points to some loss of electrolytes at any rate in the early stages (McClendon, 1915; Krogh *et al.* 1938; Picken & Rothschild, 1948), but the work of Abelson & Duryee (1948) suggests that such losses would be small since in the ovarian egg of the frog only 10% of the Na ions were found to be freely exchangeable.

Like the amphibian egg the egg of the lamprey shows—at any rate for the first 5 days—a continuous uptake of water, although the permeability of the surface of the ovum is sharply reduced in the first few hours. The reduction in the osmolar concentration is apparently accounted for by the increased water content of the egg and is of the same order as that reported in the more recent work on the egg of the frog. As judged by the loss of Cl ions, however, the permeability of the egg surface to electolytes in the first 2 days is far higher than it is in the amphibian egg.

V. SUMMARY

1. Observations have been made on the swelling of eggs in tap and distilled water. The rate of uptake of water through the outer membranes and the surface of the ovum fell off sharply within a short time of contact with water. In distilled water uptake into the perivitelline space was very much greater than in tap water, but there were no significant differences in the volume of the ovum itself in these two media.

2. The initial permeability of the ovum to water has been estimated as 0·049 μ^3/μ^2/atm./min., falling after about 7 hr. to 0·003 and remaining at this level for the first 5 days of development.

3. The outer egg membranes are apparently freely permeable to water and ions, and the mean Cl concentration of the perivitelline fluid in the later stages of development was found to be only 2·4 mM/l.

4. Evidence is presented which points to a decrease in the volume of the embryo at some point between gastrulation and hatching. In tap water a contraction of the whole egg was observed from the fifth day onwards.

5. The water content of the embryo at hatching was not very much greater than that of the ovarian egg. After hatching, until the absorption of the yolk, there was a continuous increase in the fresh weight and water content of the ammocoetes.

6. The evidence suggests that the ovarian egg is in osmotic equilibrium with the peritoneal fluid (mean 110 mM/NaCl/l.). During development the osmolar concentration of the total egg fluid was reduced to values similar to those calculated from the known dilution, i.e. 30–40 mM/l. At hatching, the osmolar concentration of the embryo (80 mM/l.) was raised within a few days to 11–120 mM/l.

7. The observed reduction in Cl concentration of the whole egg was much greater than that calculated from the increases in volume. The Cl concentration of the embryo itself was reduced from an initial value of 56 to 15 mM/l. embryo fluid a week before hatching.

8. Determinations of the total Cl content of whole eggs and embryos showed a very marked loss of Cl ions in the two days following fertilization. Within 35 days after hatching the Cl level had been raised by active uptake to about four times the values at the time of hatching.

I am indebted to many of my colleagues for their constant readiness to help in the course of this work and to the Principal and Governors of the Bristol College of Technology for the provision of the facilities for this research. I should also like to thank Dr J. D. Robertson and Mr D. A. Norton, both of whom were good enough to read and criticize my manuscript.

REFERENCES

ABELSON, H. & DURYEE, W. R. (1948) Permeability of frog egg to radioactive ions of a Ringer solution. *Anat. Rec.* **101**, 636, 653–4.

BACKMAN, E. L. & RUNNSTRÖM, J. (1912). Der osmotische Druck während der Embryonalentwicklung von *Rana temporaria*. *Pflüg. Arch. ges. Physiol.* **148**, 287–345.

BACKMAN, E. L. & SUNDBERG, C. G. (1912). Der osmotische Druck bei Rana temporaria während der Entwicklung nach dem Ausschlupfen der Embryonen. *Pflüg. Arch. ges. Physiol.* **146**, 212.

BATAILLON, E. (1900). Recherches expérimentales sur l'évolution de la Lamproie (*Petromyzon planeri*). *C.R. Acad. Sci., Paris*, **130**, 1413.

BIALASZEWICZ, K. (1908). Beitrage zur Kenntnis der Wachstumsvorgange bei Amphibienembryonen. *Bull. Acad. Sci. Cracow.*, Math. Nat., p. 783.

BRIGGS, R. W. (1939). Changes in density of the frog embryo (*Rana pipiens*) during development. *J. cell. Comp. Physiol.* **13**, 77–89.

BROWN, M. G. (1941). Collapse of the archenteron in embryos of *Amblystoma* and *Rana*. *J. Exp. Zool.* **88**, 95–105.

CAMBAR, R. (1947*a*). Début du fonctionnement du pronephros chez l'embryon de Grenouille. *C.R. Soc. Biol., Paris*, **141**, 752–4.
CAMBAR, R. (1947*b*). Valeur et modalités du transit de l'eau les jeunes larves d'amphibiens. *C.R. Soc. Biol., Paris*, **141**, 761–3.
DEMPSTER, W. T. (1933). Growth in *Amblystoma punctatum* during the embryonic and early larval period. *J. Exp. Zool.* **64**, 495–511.
GALLOWAY, T. M. (1933). The osmotic pressure and saline content of the blood of *Petromyzon fluviatilis*. *J. Exp. Biol.* **10**, 313–16.
GRAY, J. (1932). The osmotic properties of the eggs of the trout (*Salmo fario*). *J. Exp. Biol.* **9**, 277–99.
HARDISTY, M. W. (1956). Some aspects of osmotic regulation in lampreys. *J. Exp. Biol.* **33**, 431–47.
KROGH, A., SCHMIDT-NIELSEN, K. & ZEUTHEN, E. (1938). The osmotic behaviour of frog's eggs and young tadpoles. *Z. vergl. Physiol.* **26**, 230–8.
KROGH, A. & USSING, H. H. (1937). A note on the permeability of trout eggs to D_2O and H_2O. *J. Exp. Biol.* **14**, 35–7.
KUSA, M. (1952). Osmotic pressure of *Hynobius* egg throughout development. *Trans. Sapp. Nat. Hist. Soc.* **18**, 65–9.
LOEB, J. & WASTENEY, H. (1915). Note on the apparent change of the osmotic pressure of cell contents with the osmotic pressure of the surrounding solution. *J. Biol. Chem.* **23**, 157–62.
MANERY, J. F. & IRVING, L. (1935). Water changes in trout eggs at the time of laying. *J. Cell. Comp. Physiol.* **5**, 457–63.
MCCLENDON, J. F. (1915). The preservation of the life of the frog egg and the initiation of development by increase in permeability. *Amer. J. Physiol.* **38**, 163–72.
PICKEN, L. E. R. & ROTHSCHILD, LORD (1948). Vapour pressure changes in the frog's egg at fertilization. *J. Exp. Biol.* **25**, 227–36.
RICHARDS, O. W. (1940). The capsular fluid of *Amblystoma punctatum* eggs compared with Holtfreter's and Ringer solutions. *J. Exp. Zool.* **83**, 401–6.
RUNNSTRÖM, J. (1920). Über osmotischen Druck und Eimembranfunktion bei den Lachsfischen. *Acta zool., Stockh.*, **1**, 321.
SVETLOV, P. (1929). Entwicklungsphysiologische Beobachtungen an Forelleneiern. *Roux Arch. EntwMech. Organ.* **114**, 771.
VERNIDUB, M. F. & LEYZOROVITCH, H. A. (1950). The water exchange in the developing eggs of fishes. *C.R. Acad. Sci. U.R.S.S.*, n.s., **72**, 417–19.
WHEELER, M. W. (1899). The development of the urinogenital organs of the lamprey. *Zool. Jb.* **13**, 1.
YAMAMOTO, T. (1952). Osmotic pressure and cleavage in the egg of the brook lamprey *Lampetra reissneri*. *Annot. zool. jap.* **25**, 1–7.

THE CHLORIDE REGULATION OF THE BRACKISH AND FRESH-WATER RACES OF *MESIDOTEA ENTOMON* (L.)

By A. P. M. LOCKWOOD and P. C. CROGHAN

Department of Zoology, University of Cambridge, and Biophysics Unit, University of Edinburgh

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INTRODUCTION

The Isopod *Mesidotea* (*Chiridothea*) *entomon* occurs as a glacial relict in the Baltic and a few Swedish lakes (Ekman, 1919, 1940) and a closely related form *M. siberica* is found in the Arctic Ocean. According to Bruun (1924) *M. entomon* and *M. siberica* are the same species. That members of an allegedly single species should occur in both fresh and sea water is most unusual. It is clear that a comparative study of the physiology of individuals from the different habitats might be expected to give information about the relation between the various races and the mechanisms involved in the adaptation to fresh water. The evolutionary aspect of this adaptation is of particular interest as the fresh-water habitats of *Mesidotca* in Sweden have only appeared since the end of the last Ice-age.

The only previous work on the osmotic regulation of *Mesidotea* is that of Bogucki (1932), who studied the chemical composition of the haemolymph of *Mesidotea* from the Polish coast. He found that in dilute sea water the chloride concentration of the haemolymph was distinctly greater than that of the medium, but was approximately the same as that of the medium when the latter approached the concentration of pure sea water. His animals could not tolerate transfer to fresh water.

The purpose of the present investigation was to make a comparative study of the haemolymph chloride concentration of animals from fresh and brackish water both in their normal media and in media of various salinities.

MATERIALS AND METHODS

Mesidotea occurs throughout the Baltic and in six Swedish lakes. Specimens were collected from depths of 15–25 m. by the use of a small iron-framed trawl during the months of July and August 1956. Brackish-water animals were caught off Trelleborg in the south Baltic and also near Fågelsundet at the entrance to the Gulf of Bothnia. Fresh-water animals were obtained from Lake Vättern and the Lilla Ullevifjärden, a northern branch of Lake Mälaren. Most of the animals caught were in the size range 20–35 mm.

The specimens were kept in their natural medium or in experimental media made by diluting either Baltic sea water or a stock of previously concentrated

Plymouth sea water. They were fed occasionally on fragments of *Mytilus* or other suitable material.

The animals from the south Baltic were kept at 5° C. All the others were kept at room temperature.

Acclimatization was carried out in a series of stages, several days being allowed in each medium before haemolymph was taken or before the animals were transferred to the next stage.

In order to collect haemolymph the animals were first dried with filter-paper, and a micro-pipette was inserted into the haemocoel through a dorsal intersegmental membrane in the posterior thoracic region. The haemolymph was stored under liquid paraffin in a lacquered watch glass. A jelly-like clot often developed, but it was possible to obtain the serum by squeezing the clot with a needle.

The haemolymph chloride concentration was determined by electrometric titration using the first method of Ramsay, Brown & Croghan (1955). Titrations were carried out in duplicate or triplicate on samples from single animals and also on samples of the medium. The values obtained were compared with those given by the same volume of a known concentration of NaCl. The standard deviation of a series was about $\pm 1\%$. The concentration of the fresh-water media quoted below are only approximate because the accuracy of the method is much less when very dilute solutions are used.

RESULTS

The results are plotted and summarized in Fig. 1.

Trelleborg animals

The mean chloride concentration of the haemolymph was 335 mM./l. The range of variation for seven animals was 315–360 mM./l. The two readings marked with a query on the graph are not included in the above, as it was suspected that these animals had been crushed or otherwise damaged when caught.

The chloride concentration of Trelleborg sea water was 122 mM./l.

Three animals were transferred directly to full strength Plymouth sea water and were still active and apparently normal after a week. In this medium the haemolymph chloride concentration was very close to that of the sea water, but in all more dilute media tested it was definitely higher. It was possible to acclimatize the animals by several stages to approximately 10% Trelleborg sea water (14 mM./l. Cl), but when twelve animals were transferred to fresh water (Lund tap water, about 1·7 mM./l. Cl) many soon died. After three days all but one were dead or moribund. The haemolymph chloride of two of these moribund animals was measured. The values were 54 and 49 mM./l. Cl, indicating that a very considerable loss of chloride had occurred. Two other moribund animals were then transferred to Trelleborg sea water. After 3 hr. both had fully regained their normal activity and their haemolymph was found to contain 188 and 174 mM./l. Cl respectively. The only animal that was still active on the third day in tap water was moribund by the fifth day. Its haemolymph chloride had fallen to 89 mM./l. This animal

was transferred to Trelleborg sea water and had recovered normal activity after 4 hr. On re-sampling the haemolymph a chloride concentration of 195 mM./l. was found. These results indicate that a very rapid active uptake of chloride is possible, but that this mechanism can operate effectively only in media containing

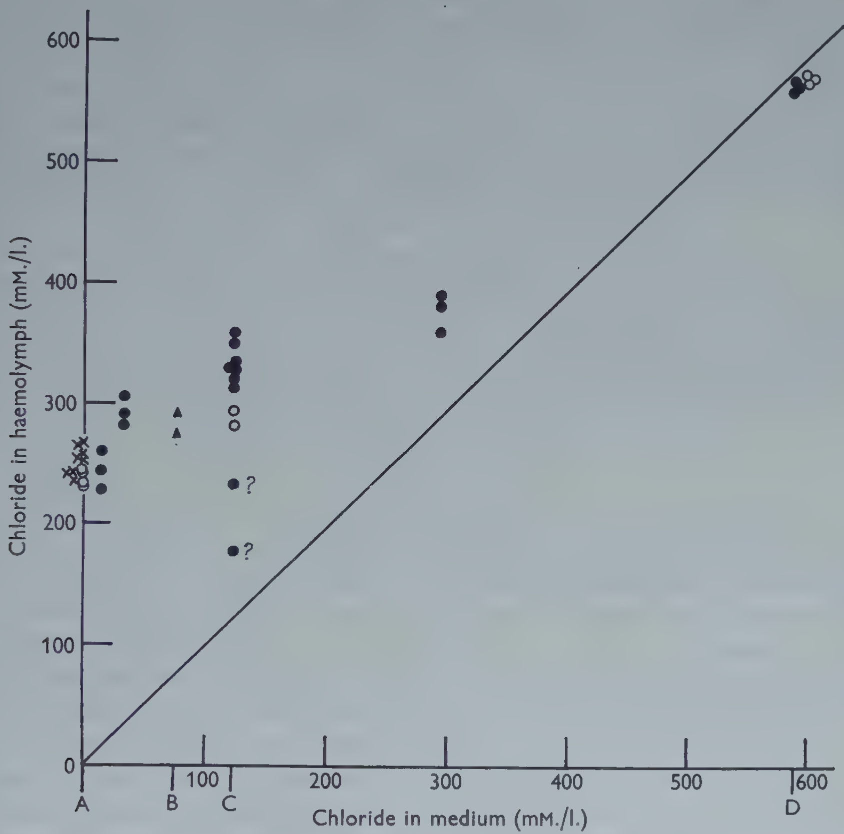

Fig. 1. The relation between the chloride concentration of the haemolymph and that of the medium. Each point represents a sample from a single animal. The diagonal represents equality of concentration. Baltic animals: from Trelleborg, ●; from Fågelsundet, ▲. Freshwater animals: from Vättern, ○; from Mälaren, ×; (? see text). A, fresh water; B; Fågelsundet sea water; C, Trelleborg sea water; D, Plymouth sea water.

appreciably more chloride than does fresh water. A very rough estimate of the rate of uptake can be made by taking the average weight to be 0·50 g. and assuming the haemolymph to be 50% of the body weight; this gives a rate of uptake of 6·5–11 μM./animal/hr. or 13–22 μM./g./hr.

Fågelsundet animals

The mean chloride concentration of the haemolymph was 285 mM./l. (two animals, respectively 276 and 294 mM./l. Cl).

The chloride concentration of the Fågelsundet sea water was 75 mM./l. Five animals were transferred directly to fresh water and after 2 days four were dead. The survivor was found to have a haemolymph chloride of only 110 mM./l.

Lake Vättern animals

The mean chloride concentration of four animals was 239 mM./l. (range 234–245 mM./l.)

The Lake Vättern water had a chloride concentration of about 0·2 mM./l. Animals were acclimatized by stages to full strength Plymouth sea water. After 5 days they were still apparently normal and the haemolymph chloride of the three sampled was very close to that of the medium.

Lake Mälaren animals

The mean chloride concentration of the haemolymph was 253 mM./l. (eleven animals, range 237–266 mM./l.).

The chloride concentration of Lake Mälaren water was about 0·5 mM./l.

No further experiments were performed on these animals.

DISCUSSION

The main interest of the present study on *Mesidotea* lies in the information it may give about the evolution of a fresh-water animal from brackish water ancestors. The evolution of *Mesidotea* as an inhabitant of fresh water is of particular interest, since its fresh-water habitats in Sweden are post-glacial and it must therefore have evolved relatively recently. It is very much more restricted in its fresh-water distribution than are the other glacial relicts. In Sweden it is confined to a few lakes across the central lowlands and this fact might also suggest a late origin.

The Baltic *Mesidotea* has evolved as a brackish-water animal, maintaining in dilute sea water the high haemolymph concentration characteristic of brackish-water Crustacea. The animal can be adapted to very dilute media, and occurs even in the northern Baltic, but could not be acclimatized to fresh water—at least, not in the time available for the present study. There is thus a clear physiological distinction between the brackish-water (Baltic) and the fresh-water races. On morphological grounds, Ekman (1919) regarded the form from Vättern as being distinct (*M. entomon* f. *vetterensis*).

The haemolymph chloride of the fresh-water race is also high and is comparable with that found in *Eriocheir sinensis* in fresh water by Scholles (1933) and in the fresh-water race of *Gammarus duebeni* by Beadle & Cragg (1940*b*). The fresh-water race of *Mesidotea* can be adapted to brackish waters and possesses the ability, remarkable in an animal normally passing its entire life in fresh water, to survive in full strength sea water. In saline waters the haemolymph chloride concentration of the fresh-water race is similar to that of the Baltic race.

It appears that the basic osmotic physiology of the two races is remarkably similar. The difference is that the fresh-water race has developed a more effective

osmo-regulatory mechanism that enables it to maintain the high haemolymph concentration of the brackish-water race even in pure fresh water. This is similar to what has occurred in the evolution of the fresh-water race of *Gammarus duebeni* (Beadle & Cragg, 1940*b*; Beadle, 1943).

Beadle & Cragg (1940*a*) and Beadle (1943) have regarded a high haemolymph concentration in fresh water as indicating an early stage in the evolution of a fresh-water animal. Potts (1954) points out that this could only be so in animals with low permeabilities. In Crustacea a low permeability would be expected as a result of the presence of a well-developed cuticle. Both the high haemolymph concentration and the ability of the fresh-water *Mesidotea* to survive in full strength sea water may be regarded as primitive features. The physiological evidence is thus in keeping with the other evidence for a recent origin of the fresh-water race.

The nature of the adaptation to fresh water in *Mesidotea* was not studied. It may be due to decreased permeability, to a more effective ion uptake mechanism, or possibly even to the production of a hypotonic urine. The brackish-water race has a powerful active uptake mechanism maintaining the haemolymph concentration hypertonic to brackish waters, and an increase in the efficiency of this might be a factor in the adaptation to fresh water. A comparative study of the permeability and active uptake of the two races would be of considerable interest.

SUMMARY

1. *Mesidotea entomon* (L.) is found in the Baltic and in certain fresh-water lakes in Sweden. It is believed that colonization of fresh water in this region has taken place since the last Ice-age.

2. In the present work animals from brackish and fresh-water habitats have been compared both in respect of the concentration of chloride in their haemolymph and of their ability to survive in media of various salinities.

3. Both fresh-water and Baltic animals have been found able to survive in Plymouth sea water, the concentration of chloride in their haemolymph being close to the concentration of chloride in this medium.

4. Baltic animals could not be acclimatized to fresh water.

5. Animals from both habitats have the same general level of chloride concentration in their haemolymph when acclimatized to dilute sea water.

6. These results are discussed in relation to the evolution of a fresh-water race from a brackish-water race.

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REFERENCES

BEADLE, L. C. (1943). Osmotic regulation and the faunas of inland waters. *Biol. Rev.* **18**, 172.

BEADLE, L. C. & CRAGG, J. B. (1940*a*). Studies on adaptation to salinity in *Gammarus* spp. *J. Exp. Biol.* **17**, 153.

BEADLE, L. C. & CRAGG, J. B. (1940*b*). Osmotic regulation in fresh-water animals. *Nature, Lond.* **146**, 588.

BOGUCKI, M. (1932). Recherches sur la régulation osmotique chez l'isopod marin, *Mesidotea entomon* (L). *Arch. int. Physiol.* **35**, 197.

BRUUN, A. F. (1924). On *Chiridothea entomon* (L.) in the southern and western Baltic. *Publ. Circ. Cons. Explor. Mer*, no. 83.

EKMAN, S. (1919). Studien über die marinen Relikte der nord-europäischen Binnengewässer. IV. Die morphologischen Folgen des Relicktwerdens. *Int. Rev. Hydrobiol.* **8**, 477.

EKMAN, S. (1940). Die schwedische Verbreitung der glazial-marinen Relikte. *Verh. int. Ver. Limnol.* **9**, 37.

POTTS, W. T. W. (1954). The energetics of osmotic regulation in brackish- and fresh-water animals. *J. Exp. Biol.* **31**, 618.

RAMSAY, J. A., BROWN, R. H. J. & CROGHAN, P. C. (1955). Electrometric titration of chloride in small volumes. *J. Exp. Biol.* **32**, 822.

SCHOLLES, W. (1933). Ueber die Mineralregulation wasserlebender Evertebraten. *Z. vergl. Physiol.* **19**, 522.

ROTATION EXPERIMENTS WITH BLIND GOLDFISH

By F. R. HARDEN JONES
Fisheries Laboratory, Lowestoft

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INTRODUCTION

Fish can maintain their orientation when rotated at constant velocity on a turntable (Steinmann, 1914; Schiemenz, 1927) in the absence of a moving visual field and when there is no relative motion between the water and the sides of the tank in which they are swimming. The response is not affected if the lateral lines are cut (Dijkgraaf, 1934), and it seems most likely that the stimulus to which the fish reacts is labyrinthine in origin. Gray (1937) suggested that the semicircular canals are the sensory channels through which a fish detects a rotation at constant velocity, and if this is so the mechanical stimulus must be an angular acceleration. But it has not been shown how a fish can be subjected to an angular acceleration during rotation at constant velocity, neither is there any experimental evidence to rule out the possibility that the centrifugal force developed during rotation, which would be detected by the otoliths, is the stimulus to which the fish responds. The behaviour of blind goldfish on the turntable was therefore re-examined.

MATERIAL

The experimental work was carried out over a period of 18 months with six small goldfish, *Carassius auratus*, 4–6 cm. in length, which were blinded by removal of their eyes while under anaesthesia. All survived the operation and subsequently grew faster and appeared to be in slightly better condition than the stock of normal fish from which they were taken. In the middle of the experiments one fish leapt from its dish on to the floor, while being returned to the aquarium and had to be killed. There were no other losses.

The reactions of blind goldfish to light. The possible sensitivity of blind goldfish to light was investigated by comparing their locomotory activity under low red light with that observed when a tungsten filament lamp giving an intensity of 1400 m.c. was switched on. As no change in activity was observed, it was concluded that blind goldfish are probably insensitive to light.

The swimming of blind goldfish. The experimental tank used in many of the experiments was a six-sided Perspex container, 30 cm. square and 15 cm. deep. The tank was completely filled with water and the fish introduced through a small hole which was afterwards closed by a rubber bung. Blind goldfish appear to be more active than normal fish, and observations showed that three of them generally swam in an anticlockwise direction. Similar behaviour was observed in an open circular tank

90 cm. in diameter. Spencer (1939) noticed this tendency to swim in one direction in normal unblinded goldfish and it could, unless allowed for, be a source of error when working at rotational speeds close to the threshold level.

APPARATUS

The turntable used was mounted on ball and thrust races and driven by a 0·3 h.p. high-speed motor rated at 1420 rev./min., final rotational speeds being obtained with the use of a 95·1 worm reduction gear and a series of pulleys. For most of the experiments two T bars were bolted to the turntable top, as shown in Fig. 1. The

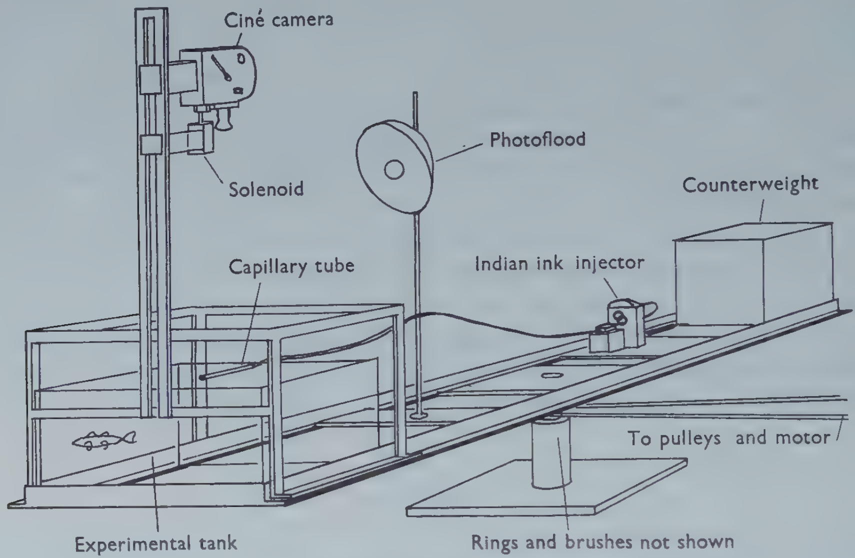

Fig. 1. Turntable used in the experiments.

T bars provided an extension of the turntable 2·4 m. in diameter and allowed the Perspex tank to be mounted either at the centre of rotation or at the periphery where the angular velocity would be the same but the centrifugal force about 8 times as great. Cinematographic records of the fish's movements during rotation were taken by a 16 mm. Bolex Paillard camera mounted above the tank as shown in Fig. 1. The camera was clockwork-driven and operated by a solenoid, and its frame speed at various settings checked against a disk turning at a known rate. When ciné films were taken a uniform field of illumination was provided by a photoflood mounted on the turntable. The electric supply to the photoflood and solenoid was led on to the table by means of rings and carbon brushes not shown in the figure.

Rotation of the turntable was started gradually and could be controlled by a clutch mechanism. When the turntable started to move a swirl was set up in the experimental tank due to the inertia of the mass of water. The presence of the swirl could be detected by injecting drops of indian ink into the tank by remote control. The injection mechanism, mounted on the T bars, was a low geared electric motor

so arranged as to press home the plunger of a hypodermic syringe, which was connected by tubing to an ink-filled capillary tube projecting into the experimental tank. At an angular velocity of 69°/sec. it was found that all relative movement between the water and the tank ceased within 4 min. of the start of rotation. A drop of indian ink injected into the tank then sank to the bottom with no indication of a swirl. To be sure that all swirls and eddies had gone, no photographic records were taken during the first 5 min. of rotation and to avoid the possibility of fatiguing the fish none were taken more than 10 min. after the start of an experiment. At lower angular velocities, down to 1·5°/sec., circular tanks or baths were used in place of the small Perspex tank, and in such experiments every precaution was taken to ensure that all relative motion between the water and the sides of the tank had ceased before observations were made. At the lower speeds 15 min. were allowed for the water to gain the speed of the tank.

The angular velocity of the turntable was found with a stop-watch but when a more accurate result was needed the data were extracted from ciné films. These showed that the speed of rotation was reasonably steady and usually did not vary by more than 0·5° in $\frac{1}{26}$ sec. Occasional variations as great as 1·5° in $\frac{1}{26}$ sec. were observed but it is difficult to say to what extent these were due to errors of tracing and measurement. It could be argued that the accelerations and decelerations accompanying these velocity changes provide the fish with information about the speed and direction of rotation and that there is no need to look for any other stimulus. This point will be dealt with later when considering the magnitude of the accelerations involved and the probable threshold level for the fish, and it will be shown that it is unlikely that any information would have been gained from the turntable itself.

PRELIMINARY OBSERVATIONS

Preliminary observations were made with single fish in the enclosed Perspex tank and in open circular tanks of 90 and 42 cm. diameter. At the start of rotation the walls of the tank moved faster than the mass of water which only gradually picked up speed. During this settling down period a fish rarely swam against the direction of rotation. When clear of the sides and bottom it often allowed itself to be carried passively round in the direction the tank was moving. However, when it touched the sides there was a marked change in behaviour. If the fish was being carried round tail first with the water it turned to head in the direction of rotation and swam rapidly with the tank at a speed close to that at which it was rotating. A fish carried passively head first with the water swam rapidly forwards when coming into contact with the sides of the tank. This reaction became less marked as the speed of the water approached that of the walls. The fish then settled down to swim against the direction of rotation, maintaining, for long periods, the same orientation or bearing relative to earth. During prolonged rotation at constant velocity a fish would often, for no apparent reason, become very excited, swimming rapidly with or against the movement of the turntable. These bursts of rapid swimming might last for 30 sec. or more, the fish then settling down to steady swimming and turning against the

direction of rotation. When the turntable was stopped, the water continued in the direction of rotation and would at times carry the fish round with it. Here again there was a marked change in behaviour if the fish happened to touch the sides of the tank. The fish then swam strongly into the swirl, maintaining its position relative to the side of the tank, until the water slowed down.

These observations are similar to those made by Dijkgraaf (1934) on the reactions of blinded minnows to rotations at constant velocity. There can be little doubt that the behaviour of the fish observed at the beginning and end of the experiment is stimulated by contact with the sides, the movements of the fish being such as to maintain its position relative to the surrounding tank. It is only when the water has gained the speed of the tank that the response to rotation is observed. The fish then reacts by making well-defined turning movements which enable it to maintain more or less the same geographical bearing, and it will, at an angular velocity of 69°/sec., keep this up for 1–2 min. at a time. An analysis of cinematographic records taken at this speed of rotation showed that there was no significant quantitative difference in the response of the fish, as measured by its own average angular velocity, when the experimental tank was at the centre or the periphery of the turntable. These results are summarized in Table 1.

Table 1. *Angular velocity of blind goldfish swimming in the direction opposite to that of rotation at 69°/sec. in the two positions on the turntable*

Fish ...	1		2		4	
Position on turntable	Centre	Periphery	Centre	Periphery	Centre	Periphery
Mean angular velocity of fish in°/sec.	69·8	73·5	62·3	67·6	60·7	59·8
Standard deviation	5·6	14·2	17·7	14·3	16·5	14·8
No. of complete turns through 360° used to estimate angular velocity	21	27	26	21	27	21
t test, value of P	0·2–0·3		0·3		0·8–0·9	

Other observations were made to determine the lowest speed of rotation at which a response would be observed. These experiments were carried out with single fish in a circular tank, 90 cm. in diameter. The circular tank was rotated for 15 min. to allow the water to pick up the speed of the turntable. A fish was then gently put into the water and left for 5 min. to settle down. During the next 5 min. the time that the fish spent swimming clockwise was recorded on a stop-watch. The turntable was then stopped and the fish removed. The motor was reversed and the tank rotated for a further 15 min. in the opposite direction. The same fish was then put into the tank and the time that it spent swimming clockwise again recorded. If the fish was responding to the rotation there should be an appreciable difference in the time that it spent swimming clockwise at anticlockwise and clockwise rotations. This method gave a simple and objective measure of the strength of the response

and avoided any errors that might arise from the fish having a bias to swim in one particular direction.

The results of these observations are summarized in Fig. 2, where it will be seen that, for the best performers, the threshold angular velocity to induce a response appears to be about 3°/sec. At low angular velocities the fish did not always respond, and for some time there might be no apparent response at all, the fish swimming quietly in the tank, either at random, or, in the case of those with a bias, mainly in one direction. When a response did occur, their behaviour was markedly different.

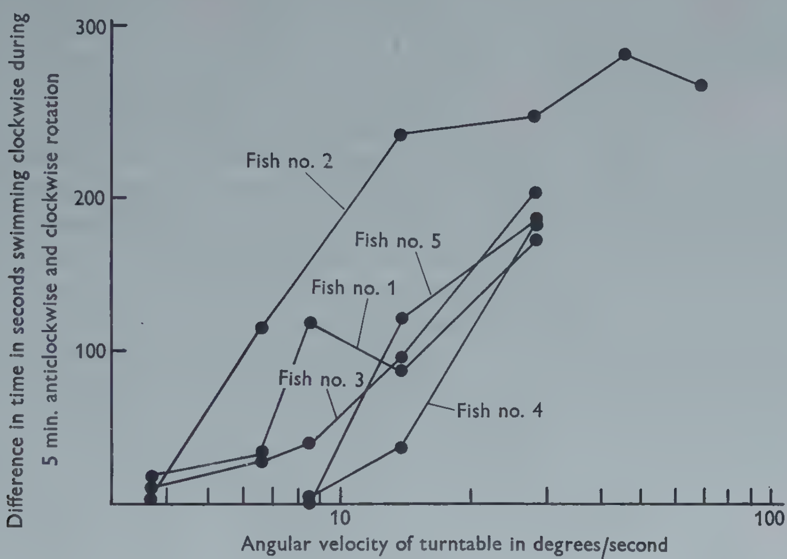

Fig. 2. Results of experiments made to determine the lowest angular velocity to which fish respond.

A fish would remain more or less poised in midwater, turning in a small circle against the direction of rotation, making distinct compensatory movements with the tail between which it appeared to lose way and was carried round with the turntable relative to the ground. A second series of observations was made in which subjective estimates were made of a fish's response to low angular velocities, with results that are summarized in Table 2. At 8·8°/sec. the best fish could maintain the same orientation for up to 30 sec., but in general a response at low angular velocities was marked by over-compensation. At the lowest angular velocity used, 1·7°/sec., a fish would sometimes appear to be 'aware' of a sensation of rotation, although frequent 'mistakes' were made in turning and the fish could never be said to show a clear positive response. While subjective judgements of this sort are always open to criticism, it will be seen that the results of the two series of experiments are in good agreement one with another.

Some experiments were also carried out to see how long a fish would continue to respond to rotation. At an angular velocity of 26°/sec. a fish was still responding well, although a little fatigued, after 2 hr., when the experiment was stopped.

Table 2. *Subjective estimates of response of blind goldfish to rotation at constant velocity in five experiments*

(+ =clear positive response, ? =possible response, – =no response.)

Angular velocity of turntable in degrees per second

Fish	1·7°					3·6°					8·8°					12·8°				
1	–	?	?	?	?	+	?	?	+	+	+	+	+	+	+			·		
2	–	–	–	–	?	+	+	+	+	?	+	+	+	+	+			·		
3	–	–	–	–	–	?	?	+	+	?	?	+	+	+	+			·		
4			·			?	–	–	–	–	–	+	?	?	?	+	lost in accident			
5			·			?	?	–	–	–	+	?	?	?	–	+	–	+	+	+

THE STIMULUS TO WHICH THE FISH RESPONDS

An attempt was made to check up on the various stimuli to which the fish might react. It was observed that the response does not develop suddenly in the early stages of rotation and only becomes clearly defined as the initial water swirl dies away. The water swirl and vibration of the motor accompanying rotation might provide the fish with some clue as to what is going on.

(1) *Water swirl and vibration of motor.* A fish was given the vibrational stimuli accompanying rotation and the water swirl appropriate to clockwise or anticlockwise movement. This was done by modifying the turntable so that the rotation of the tank about a vertical axis was changed to a curvilinear translation. The centre spindle of the turntable was drilled to clear a $\frac{3}{4}$ in. steel rod firmly attached to the base plate. At its upper free end it was connected by a chain and two sprocket wheels of equal diameter to a second turntable mounted on the T bars towards the periphery of the main one. When the latter rotated, the secondary turntable, carrying the experimental tank, turned at the same angular velocity but in the opposite direction so that the tank, water and fish were carried round along a curvilinear path without rotation. There was, of course, no water swirl due to this movement. Under these conditions of curvilinear translation no response was ever made by the fish. However, if sprocket wheels of unequal diameter were used, so that the second turntable rotated relative to the ground, the fish reacted well. A number of experiments were made in which a curvilinear translation was immediately preceded by a hand-made water swirl to simulate the conditions at the onset of normal rotation. No reaction was ever seen to suggest that the fish gained any information from the swirl or vibrations accompanying the movement of the turntable.

(2) *Accelerations due to the movement of the turntable.* The fish might react to the impulsive jerk or acceleration which occurs when the turntable gets under way and small variations in turntable speed might provide information about the velocity and direction of rotation. The first point to consider is the threshold level for the perception of an angular acceleration and the best that can be done is to assume, not unreasonably, that the fish labyrinth is at least as sensitive as that of man. With a very sensitive human subject the minimum sustained angular acceleration which gives rise to a sense of rotation is of the order of $0{\cdot}2°/\text{sec.}^2$ (Groen & Jongkees, 1948),

and it will be assumed that the threshold for a fish is similar. Now the product of the time t in seconds and the angular acceleration α in degrees/sec.^{2} required to reach the threshold for a sense of rotation is, according to Mulder's Law, constant, and Groen & Jongkees go on to show that for man its value lies between 2 and 3. Furthermore, the Mulder Product αt, as it is called, should be numerically equal to the minimal detectable impulse, which is most easily measured by determining the lowest angular velocity, in degrees per second, from which a sudden deceleration gives rise to a sense of rotation. Experiments (Dodge, 1923; Groen & Jongkees, 1948; Hulk & Jongkees, 1948) show that this minimal angular velocity is, as expected, of the order of 2–3°/sec. So it seems reasonable to conclude that an angular acceleration or deceleration of 0·2/sec.^{2} or more could be detected by a fish if the αt product is not less than 2, and that the threshold level for an impulsive stimulation is about 2–3°/sec.

To test the possible orientating effect of the initial acceleration which occurs when the turntable gets under way a constant angular velocity was reached after a period of controlled subliminal acceleration. A quantitative estimate of the fish's response was made after 5 or 10 min. rotation at the final speed. This experiment could not be carried out with the turntable described here, and I am very grateful to Dr C. S. Hallpike, F.R.S., Director of the Medical Research Council's Otological Research Unit for allowing me to use his special revolving chair (Byford, Hallpike & Hood, 1952) at the National Hospital, Queens Square, London. On this chair fish were accelerated at a little less than 0·2°/sec.^{2} to an angular velocity of 25°/sec. As the fish clearly responded at the final speed after the subliminal acceleration, the initial jerk at the start of rotation cannot be the sensory clue to which it reacts.

At an angular velocity of 69°/sec. the variations in the speed of the Lowestoft turntable were normally not greater than 0·5° in $\frac{1}{26}$ sec. but occasional variations of 1·5° in $\frac{1}{26}$ sec. were observed. The angular accelerations, 13° and 39°/sec.^{2} respectively, are well above threshold, but the αt products 0·5 and 1·5 are below although close to the critical value. Analysis of cinematographic records failed to show any correlation between movements of the fish and variations in turntable velocity. Such variations in turntable velocity are bound to be considerably if not completely smoothed out by the inertia of the water. I have myself sat on the Lowestoft turntable and, once the sensation due to the initial acceleration had died away, never experienced any feeling of rotation. Furthermore, the fish responded well on the Medical Research Council's chair which is a good deal steadier than the turntable used in Lowestoft. It does, therefore, seem most unlikely that the reactions of the fish can be due to variations in turntable velocity.

(3) *Centrifugal force.* In man the threshold level for the perception of a linear acceleration is about 0·001 G. (Groen & Jongkees, 1948) and that for a fish may be assumed to be similar. The lowest angular velocities to which a fish was found to respond lie between 1° and 10°/sec. and in the tank used in these experiments the centrifugal forces developed will have been of the order of 0·001–0·00001 G., which although below the probable threshold level, suggest that the stimulus is one that should be looked into. This was done by making a quantitative measure of the

strength of the response of the fish when at the centre or periphery of the turntable at three different angular velocities.

The results of these experiments should show whether the response varies with centrifugal force or angular velocity, the method being essentially similar to that used by Maxwell (1923) to determine whether the turning movements of the horned lizard *Phrynosoma* were made in response to angular acceleration or centrifugal force. As in the threshold experiments, an objective measure of the response was obtained by recording the difference in the time spent swimming clockwise during anticlockwise and clockwise rotations.

Results. The results of these experiments are summarized in Table 3. Whether the fish is at the centre or periphery of the turntable there is, at any particular angular velocity, no significant difference in the strength of the response despite an almost eightfold difference in centrifugal force. Furthermore, a greater centrifugal force does not by itself lead to a stronger response. This is evident when comparing the results obtained at 26° (centre) with those at 13° (periphery) and 13° (centre) with 6·5° (periphery). There can be little doubt that the response of the fish, as measured here, is related in some way to the velocity of rotation and not to centrifugal force.

(4) *Angular accelerations*. The results of the previous experiments show that the fish are not responding to the initial water swirl, variations in turntable velocity or centrifugal force. The possibility must now be examined that they are reacting to an angular acceleration, and the first problem to consider is how this could occur during rotation at constant velocity.

So long as a fish swims at an angular velocity equal and opposite to that of the rotation it is not subjected to any turning movement about its vertical axis, although it may be carried along a curvilinear path relative to the ground. Furthermore, provided the fish swims at a steady speed without rolling or pitching it is not subjected to an angular acceleration even if its own angular velocity is greater or less than that of the turntable. Under conditions of complete stability angular accelerations can only arise through variations in the fish's own angular velocity and if these occur, the fish will yaw about its vertical axis relative to the ground. This is shown diagrammatically in Fig. 3. The turntable is rotating in an anticlockwise direction at a velocity of 60°/sec. In A the fish is swimming at a constant angular velocity equal and opposite to that of the rotation. Relative to the ground the fish is carried without rotation, along a curvilinear path whose radius is equal to the difference between that of the turntable and the circle in which the fish swims relative to the water. The effect of a change in the angular velocity of the fish is shown in B. As the fish slows up it is carried backwards relative to the ground and yaws about its vertical axis in the direction of the turntable's rotation, and is thus subjected to an angular acceleration.

Variations in the fish's own swimming speed could then be a source of angular accelerations and the problem arises as to whether or not such variations occur and if they do, could the angular accelerations they lead to enable the fish to be aware of the speed and direction of rotation.

Table 3. *Results of experiments to test the possible reactions of fish to centrifugal force*

	Fish no. 1						Fish no. 2					
Angular velocity of rotation in °/sec. ...	26		13		6·5		26		13		6·5	
Position of fish on turntable	Centre	Periphery	Centre	Periphery	Centre	Periphery	Centre	Periphery	Centre	Periphery	Centre	Periphery
R in cm.	15	120	15	120	15	120	15	120	15	120	15	120
Centrifugal force $\omega^2 r$,	2·8	22·4	1·07	8·56	0·19	1·52	2·8	22·4	1·07	8·66	0·19	1·52
Number of experiments	10	10	10	10	10	10	10	10	10	10	10	10
Difference in seconds between the time that the fish was seen swimming clockwise during five minute periods of anti-clockwise and clockwise rotation	170·7	138·3	112·0	121·1	52·6	53·5	190·9	181·3	109·0	109·0	74·1	87·8
Standard deviation	69·2	58·1	36·2	37·7	48·9	49·8	37·2	44·6	53·4	37·1	38·9	51·8
t test, value of t	1·1347		−0·5506		0·0408		0·5229		0·0000		−0·6695	
D.F. = 18, value of P	> 0·2		> 0·5		> 0·5		> 0·5		—		> 0·5	

Dr J. D. Hood (Medical Research Council's Otological Research Unit) has drawn my attention to another way in which a fish could be subjected to an angular acceleration during rotation at constant velocity. When a man is rotated on the revolving chair no sensation of turning is felt once the effect of the initial acceleration has died away as so long as the head is held still. However, if one rapidly nods or tilts the head there is an immediate sensation of turning and a clear indication of the direction in which the chair is moving. The explanation of this is that movement of the head in a plane other than the horizontal could alter the position of all three pairs of canals relative to the plane of rotation and that they therefore receive an impulsive stimulation whose strength depends on the angular velocity of the chair

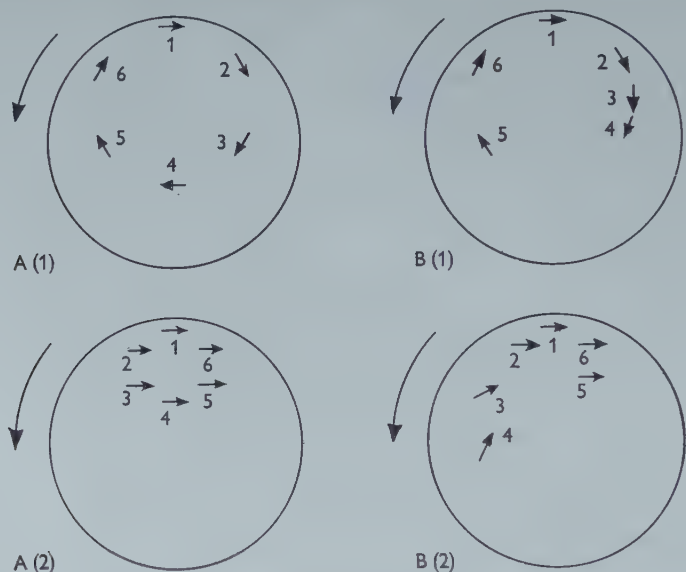

Fig. 3. A diagram to show the movements of a fish relative to the tank and ground during rotation at constant angular velocity of 60°/sec. In A(1) the fish swims at an angular velocity equal and opposite to that of the rotation and its path relative to the ground is shown in A(2). In B(1) the angular velocity of the fish decreases after 2 sec; in B(2) it can be seen that the fish is then rotated about its vertical axis relative to the ground.

and the extent of the movement of the head. If the head is held still in the new position the sensation of turning dies away in a manner similar to that experienced when the chair starts to turn. This means that if the fish is not completely stable and rolls or pitches about its longitudinal or transverse axis when swimming at an angular velocity differing from that of the turntable, the semicircular canals will be subjected to accelerations from which the fish might be able to tell the direction of rotation.

So there are two possibilities to look into. The first is whether variations in the angular velocity of the fish lead to accelerations from which it might detect the movement of the turntable, the second is whether the fish rolls or pitches when swimming to such an extent as to subject the semicircular canals to impulsive stimuli during rotation at constant velocity.

(a) *Variations in the angular velocity of the fish*

The T bars were removed from the turntable and the Perspex tank placed at its centre. Pointers attached to the spindle of the turntable and moving over a large degree scale made possible an accurate determination of angular velocity. The camera was mounted over the centre of the tank but did not rotate with it. In working up the films the negatives, or positives when printed, were projected vertically on to paper and careful tracings made of the position of the fish and the tank. From these tracings the angular velocity of the fish and tank could be found.

Results. A study of the slow motion films and a frame by frame analysis of those selected for working up showed that the fish do not swim continuously against the direction of rotation. This is evident from Fig. 4 in which the angular velocity of

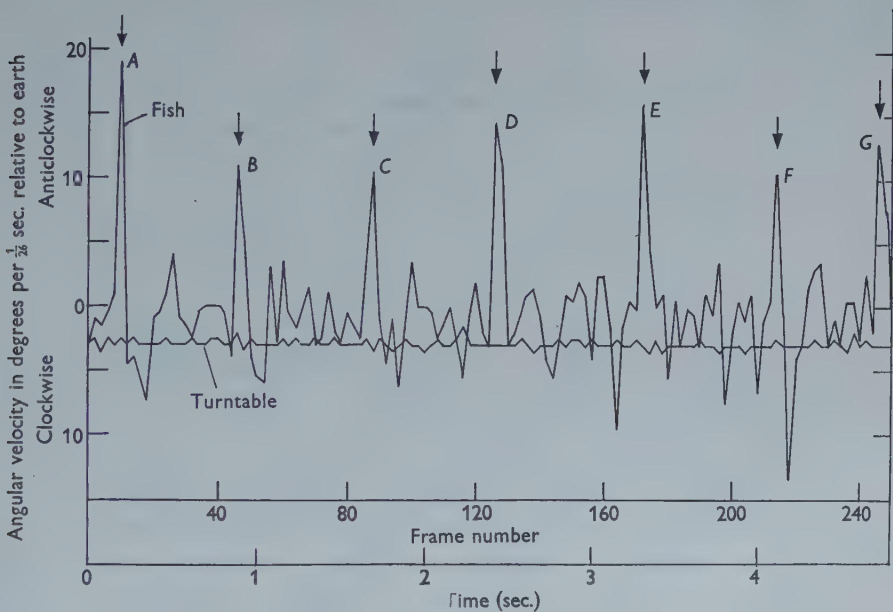

Fig. 4. Analysis of a ciné film to show angular velocity of fish and turntable relative to earth. Turning movements are indicated by arrows.

the fish and the turntable relative to earth are shown at intervals of $\frac{1}{26}$ sec. The angular velocity of the fish varied considerably. The sudden compensatory movements of 15–20° were always associated with active turning movements against the direction of rotation. The angular velocity of the fish rarely, if ever, fell away smoothly between compensatory turns. The reason for this appeared to be that the fish was using its tail, intermittently, as a rudder to compensate for the decrease in its angular velocity. In Fig. 5, the data used in Fig. 4 are presented to show the degrees turned through by the turntable and fish, after successive intervals of $\frac{1}{26}$ sec. During this particular revolution of the turntable the fish just failed to maintain its orientation, and inspection of the ciné film shows that it is losing way and being carried backwards relative to the ground.

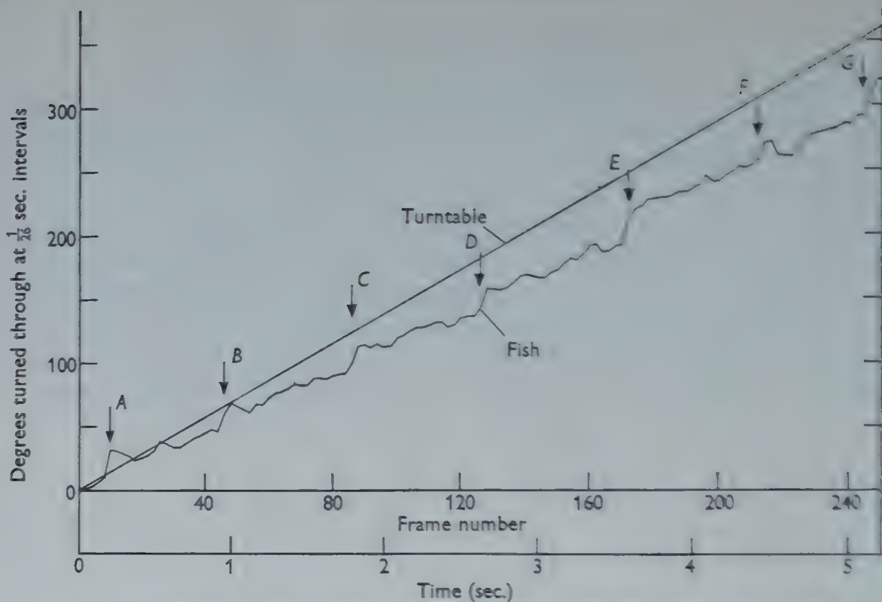

Fig. 5. Analysis of a ciné film to show the degrees turned through, irrespective of direction, at $\frac{1}{26}$ sec. intervals. Turning movements are indicated by arrows.

Fig. 6. Analysis of the movements and orientation of a fish between two compenstory turning movements. Full explanation in text.

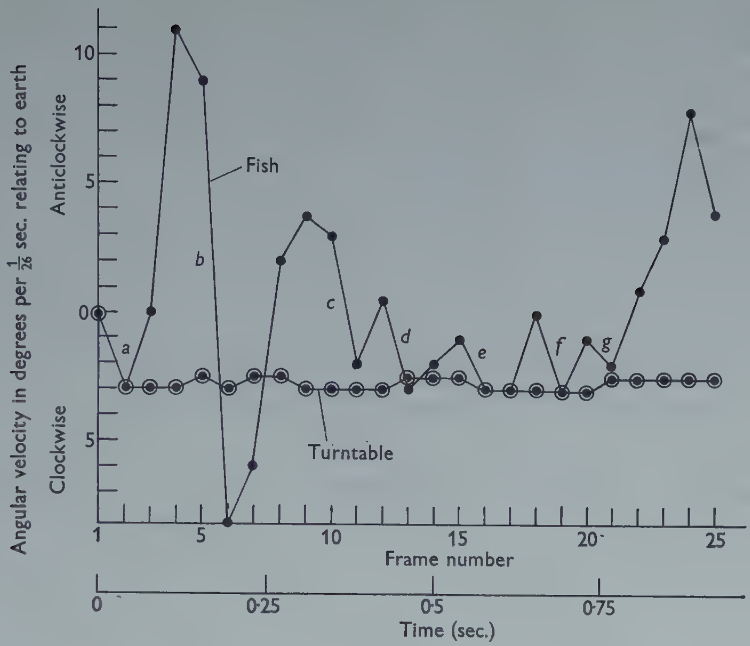

Fig. 7. Graphical presentation of the movements of the fish shown in Fig. 6. Full explanation in text.

A more detailed analysis of the movements and orientation of a fish between two turning movements is shown in Figs. 6 and 7. The film was taken when the fish was being rotated clockwise at a speed of 73°/sec. The camera was running at 52 frames/sec. and tracings of the fish were made from every other frame. In Fig. 6 the point through which the vertical axis of the fish was judged to pass has been lined up over the centre of each square so that the orientation of the fish relative to the ground can be seen easily. The velocity of the turntable is also given in Fig. 7. Taking the two figures together, the following points can be noted:

Frames	Remarks
1–2	Fish carried passively clockwise with turntable
2–3	Tail coming over at start of turn; fish holding its own relative to ground
3–5	Anticlockwise turn of 20°
5–7	Fish turns clockwise during recovery of tail
7–10	Much of the lost ground regained as the tail comes over to the left, with a rudder-like action
10–11	Fish carried clockwise with tank
11–12	Clockwise rotation checked, presumably by tail
12–13	Fish carried passively clockwise with tank, the extra 0·5° may be a drawing error
13–15	Clockwise rotation partially checked; presumably by tail although this cannot be seen on the film
15–17	Fish carried passively clockwise with tank
17–18	Clockwise rotation partially checked; slight indication of tail coming over to the left
18–19	Fish carried passively clockwise with tank
19–21	Clockwise rotation partially checked; tail coming over to the left
21–25	Fish makes an anticlockwise turn of 16°, re-gaining all the lost ground

In this sequence it will be seen that between frames 10 and 21, after which the next turn develops, the fish does not make any ground against the clockwise rotation and only succeeds in checking, from time to time, its movement with the turntable. So far as the fish's own movements are concerned, it is evident that rotation proceeds in a series of angular accelerations and decelerations, and the question now arises as to whether they can give any information about the speed and direction in which the turntable is moving.

Although calculations, summarized in Table 4, show that all but one of the decelerations occurring in the cycle analysed in Figs. 6 and 7 could probably have been detected, it is difficult to see what useful information they could give to the fish. The mechanical stimuli it receives will be identical to that which it would get if it were swimming anticlockwise at a variable angular velocity when the tank was still.

Table 4

Deceleration	Duration t in sec.	Changes in degrees	α degrees/sec.^{2}	αt (impulse)
a	0·031	3	78	3
b	0·031	17	441	17
c	0·031	5	130	5
d	0·031	3·5	91	3·5
e	0·031	2	52	2
f	0·031	3	78	3
g	0·031	1	26	1

If it slows up, there will be an increase in the resting discharge from the right semicircular canal, but these re-afferent stimuli must be ignored (cf. von Holst, 1954) otherwise the fish would circle for ever. The fact that the fish may be swimming at a variable angular velocity in a rotating tank cannot alter the mechanical stimuli received in the horizontal canals, although the fish may be accelerated backwards relative to earth as it slows down. As Lowenstein & Sand (1936) remark: 'the arrest of rotation is physically equivalent to acceleration in the opposite direction'. So although variations in the angular velocity of the fish do occur, and although it seems probable that accelerations introduced in this way could be perceived by the fish, it seems that they cannot provide any information about the rotation of the turntable.

(*b*) *The detection of rotation by rolling or pitching*

A fish might be able to detect the rotation of the turntable if it rolls or pitches when it swims at any angular velocity which is not both equal and opposite to that of the rotation. The rolling or pitching need not be deliberate and might be no more than normal instability during locomotion. If, during rotation at constant velocity about a vertical axis, the fish rolls, the anterior vertical canals will receive impulsive stimuli as they are brought into the plane of rotation and the horizontal canals will be stimulated as they are taken out of it. During pitching the posterior vertical and horizontal canals will be involved. It is possible to calculate the order of magnitude of the roll or pitch necessary to detect rotation at various constant

angular velocities. During a roll or tilt, the force acting on the anterior vertical canal will be proportional to the product of the sine of the angle of tilt and the angular velocity, while that of the horizontal canal will be proportional to the product of the cosine of the angle of tilt and the angular velocity. According to Egmond, Groen & Jongkees (1949) the minimal detectable cupula deflexion is about 0·25° and the magnitude of the cupula deflexion obtained during impulsive stimulation is of the order of one-tenth of the angular velocity from which the subject or preparation is brought to rest. So considering each canal separately, the minimal angle of tilt required to give a sense of rotation at constant velocity will be $\sin\theta = (0{\cdot}25 \times 10)/V$ for the anterior vertical canals, and $\cos\theta = (0{\cdot}25 \times 10)/V$ for the horizontal canals,

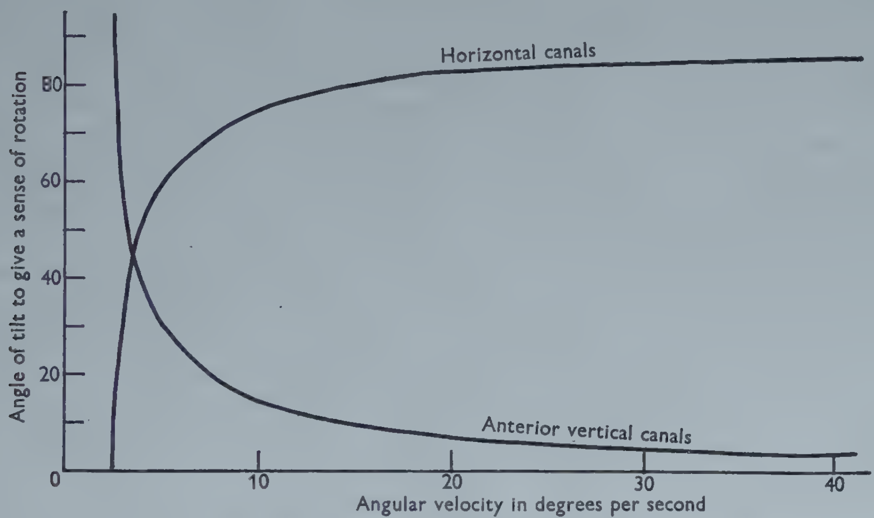

Fig. 8. The angle of tilt required to give a sensation of turning during rotation at different angular velocities.

where V is the angular velocity of rotation in degrees per second. The values obtained by calculation from these equations are shown in Fig. 8. The critical angles for pitching would of course be similar. If the sensitivity of the fish labyrinth is of the same order as that of man, and rolling or pitching is the means by which they are able to detect and respond to rotations at constant velocity, it should be possible to observe movements of the order given in Fig. 8 at different angular velocities.

Attempts to measure the roll of a fish while it was responding to rotation at constant velocity have not been successful. The ciné camera was mounted vertically above the tank, and a mirror placed so as to give an end on picture of the fish on half of the field of view. At rotations below 20°/sec. the fish headed into the mirror for only a few seconds at a time and inspection of films taken at this and higher angular velocities failed to reveal any sign of rolling. The rolling should, of course, be greatest at lower angular velocities but, if confined in a small tank, the fish then spends most of its time swimming along the sides, and in a larger tank the fish is

hardly ever in the field of view of the camera. Some head on shots were made of fish swimming in a stationary tank but no rolling movements could be detected. The fish appeared to be quite stable. Careful observations on fish in large circular tanks being rotated at low angular velocities did not give any indication of any rolling or pitching. Negative results do not, of course, exclude the possibility that the fish becomes aware of the rotation by rolling or pitching. Small movements of this sort are difficult to detect, and if the threshold for perception of impulsive stimuli is lower than has been supposed their observation and measurement will not be easy.

DISCUSSION

It seems clear enough that the reaction of blind fish to rotation at constant velocity is a real one and that the stimulus to which the fish responds is neither one of contact, a water current, centrifugal force nor the initial acceleration when the turntable gets under way. It does, however, seem most likely that the semicircular canals are the sensory channels involved. While the decelerations to which the fish is subjected between compensatory swimming movements are of such a magnitude and duration which makes it reasonable to suppose that the fish could detect them, it is very difficult to see how they can provide any useful information as to the direction or speed of rotation. Hood's suggestion that the fish detects the rotation as it rolls or pitches slightly during swimming is the more attractive hypothesis as it has a foundation in human experience: it is certainly possible. But at the moment the experimental evidence is not sufficient to decide between these two. The next line of approach must inevitably involve some operations on the labyrinth and perhaps an electrophysiological investigation into the sensitivity of the canals of the fish labyrinth to tilting or pitching during rotation at constant velocity.

It remains to be seen how far a fish's ability to maintain its orientation when subjected to rotation at constant velocity about a vertical axis might enable it to maintain a steady course in natural conditions. If it is assumed that the threshold rotation is about 3°/sec. the diameter of the swirl in which a fish could maintain a bearing or 'head into the stream' could be calculated for different current velocities. But the worth of such calculations depends on a detailed knowledge of small scale water movements in the sea and at the moment the necessary data do not seem to be available. At all events an orientation of this nature would depend on local hydrographic conditions, and it does seem unlikely that the response could be of any value for navigation over long distances in open water.

SUMMARY

1. Blind goldfish react to rotation at constant angular velocity by swimming against the direction of rotation so as to maintain, on the whole, the same orientation or bearing relative to earth.

2. The lowest angular velocity to which the fish appear to react is below 10°/sec. For the best performers the threshold is about 3°/sec.

3. The stimulus to which the fish responds is not one of contact, initial swirl or water current (when the turntable gets under way), variation in turntable velocity or centrifugal force.

4. The semicircular canals are probably the sensory channels through which a fish is able to detect rotation at constant velocity and the mechanical stimulus to which it responds is probably an angular acceleration. How the fish becomes aware of angular accelerations during rotation at constant velocity is not yet understood.

I am very grateful to Mr H. W. Lissmann, F.R.S., and Prof. O. E. Lowenstein, F.R.S., for their advice and criticism while I was doing this work.

REFERENCES

Byford, G. H., Hallpike, C. S. & Hood, J. D. (1952). The design, construction and performance of a new type of revolving chair. *Acta otolaryng., Stockh.*, **42**, 511–38.

Dijkgraaf, S. (1934). Untersuchungen über die Funktion der Seitenorgane an Fischen. *Z. vergl. Physiol.* **20**, 162–215.

Dodge, R. (1923). Thresholds of rotation. *J. Exp. Psychol.* **6**, 107–37.

Egmond, A. A. J. van, Groen, J. J. & Jongkees, L. B. W. (1949). The mechanisms of the semicircular canal. *J. Physiol.* **110**, 1–17.

Gray, J. (1937). Pseudo-rheotrophism in fishes. *J. Exp. Biol.* **14**, 95–103.

Groen, J. J. & Jongkees, L. B. W. (1948). The threshold of angular acceleration perception. *J. Physiol.* **107**, 1–7.

Holst, E. von (1954). Relations between the central nervous system and the peripheral organs. *Brit. J. Anim. Behaviour*, **2**, 89–94.

Hulk, J. & Jongkees, L. B. W. (1948). The turning test with small regulable stimuli. II. The normal cupulogram. *J. Laryngol.* **62**, 70–75.

Lowenstein, O. & Sand, A. (1936). The activity of the horizontal semicircular canal of the dogfish, *Scyllium canicula*. *J. Exp. Biol.* **13**, 416–28.

Maxwell, S. S. (1923). *Labyrinth and Equilibrium*, pp. 163. Philadelphia and London: Lippincott.

Schiemenz, F. (1927). Das Verhalten der Fische in Kreisströmungen und in geraden Strömungen, als beitrag zur Orientierung der Fische in der freien natürlichen Wasserströmung. *Z. vergl. Physiol.* **6**, 731–50.

Spencer, W. P. (1939). Diurnal activity rhythms in fresh-water fishes. *Ohio J. Sci.* **39**, 119–32.

Steinmann, P. (1914). Über die Bedeutung des Labyrinthes unter der Seitenorgane für die Rheotaxis und die Beibehaltung der Bewegungsrichtung bei Fischen und Amphibien. *Verh. naturf. Ges. Basel*, **25**, 212–43.

AMINO-ACID METABOLISM IN LOCUST TISSUES

By B. A. KILBY and ELISABETH NEVILLE
Department of Biochemistry, University of Leeds

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INTRODUCTION

The precise study of intermediary metabolism in insects has lagged behind corresponding studies with vertebrates and micro-organisms. The development of numerous and very active synthetic insecticides in recent years, however, has stimulated a study of fundamental insect biochemistry, since such knowledge is a prerequisite for the understanding of the biochemical mode of action of these poisons. Since one biochemical characteristic of insects is the very high concentration of free amino-acids in the haemolymph, it was thought that an investigation of some aspects of amino-acid metabolism might reveal unusual features.

Very little has been published on the intermediary metabolism of amino-acids in insects. Data tabulated by Albritton (1955) show that the same ten amino-acids which are classified as essential for the rat are also essential for six species of insects. (For a review of the literature, see Lipke & Fraenkel, 1956.) Patton (1953) refers to some experiments of Auclair (1949), in which an increased dietary intake of a particular amino-acid was followed by an increase in concentration in the haemolymph of a different amino-acid, e.g. ingested glutamic acid resulted in increased alanine in the blood, and such results would readily be explicable if transamination reactions were occurring similar to those which are well known in mammalian systems. More direct demonstrations of particular enzymic reactions *in vitro* are infrequent. Barron & Tahmisian (1948) have shown that the alanine/glutamate transamination reaction is catalysed *in vitro* by a homogenate of cockroach muscle. These workers also reported an increased rate of oxygen uptake by tissue homogenates on the addition of glutamic acid. Agrell (1949) found that methylene blue was reduced more rapidly by homogenates of *Calliphora* when glutamate, alanine, glycine or valine was added. In a review, Auclair (1953) states that he has found D-amino-acid oxidase activity in the fat-body and Malpighian tissues of three insect species but not in a fourth, and that the same tissues showed a lower L-amino-acid oxidase activity.

These and other results suggest the possibility that the interconversion and synthesis of amino-acids may well be similar in insects and mammals. The present study is restricted to an investigation into some aspects of the amino-acid metabolism in the Desert Locust (*Schistocerca gregaria* Forsk. phase *gregaria*), using preparations of fat-body and other tissues. A preliminary account of some of the results has been given elsewhere (Kilby & Neville, 1956).

MATERIALS AND METHODS

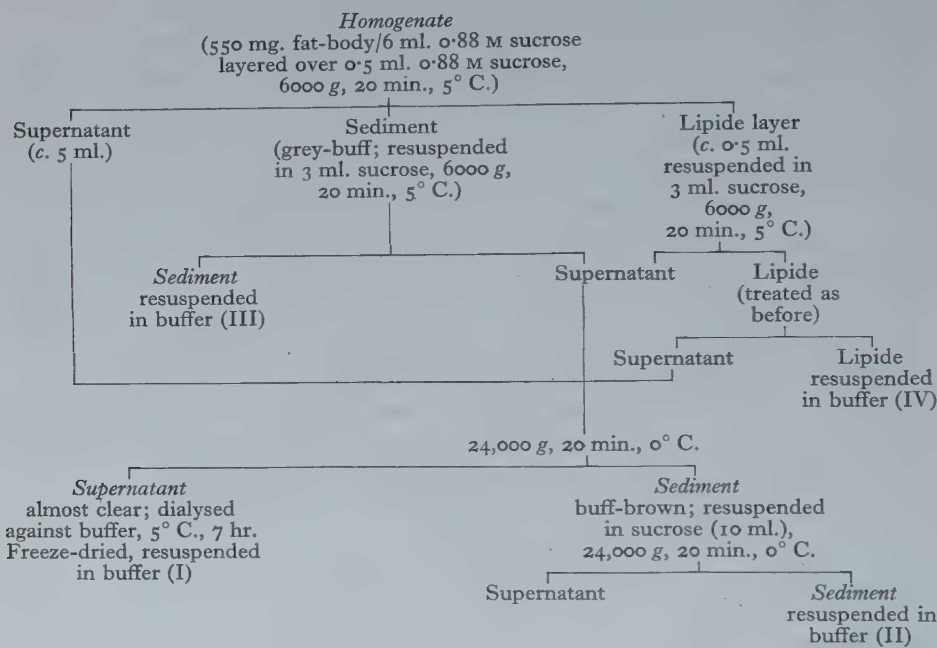
Reagents. Diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN) and cytochrome *c* were supplied by Messrs L. Light and Co. Ltd. Reduced diphosphopyridine nucleotide (DPNH) was prepared from 95 to 100% pure DPN by the method of Beisenherz *et al.* (1953) by reduction with dithionite; control experiments showed that excess dithionite was removed satisfactorily.

Experimental animals. Desert locusts (*Schistocerca gregaria*) were bred in the laboratory from a stock originally supplied by the Anti-Locust Research Centre, London. The type of cage and conditions of rearing were based on those recommended by Hunter-Jones (1956). Up to 200 insects were kept in each glass and wooden cage, which measured approximately $2 \times 2 \times 2$ ft. The temperature in the cages was allowed to vary between 21 and 38° C.; the humidity was increased by a dish of water below the perforated zinc cage floor but was not accurately controlled, and depended mainly on the amount of fresh grass present. The cages were kept in a small greenhouse, and daylight was supplemented between 6 a.m. and midnight by a 70 W. filament lamp in the roof of each cage. The food, which was given in excess, consisted of grass supplemented by an artificial diet composed of bran, dried alfalfa meal, powdered whole milk and dried brewers' yeast in the proportions of 2:2:2:1 by volume. The proportions of the two foods varied during the year, the amount of artificial diet being greater in winter when fresh grass was scarce. For experimental purposes, 5th instar hoppers were used in the 3rd to 5th day of the stadium, or adults in their 3rd to 7th day.

Tissue preparations. The insects were decapitated and the required tissues dissected out under ice-cold insect Ringer solution (Bodenstein, 1946) using a low-power binocular microscope. When large amounts of the tissue were required, the tissues were kept for up to 2 hr. in ice-cold Ringer solution, which was then decanted, and the tissue added to 10–30 vol. of buffer solution or 0·88 M sucrose. This suspension was then homogenized for the minimum time for cell breakage in an all-glass, chilled Potter–Elvejhem blender. The homogenate was dialysed, if this was required, against 200–400 vol. of buffer at 5° C. for 4–8 hr. with stirring.

Preparation of cell fractions. Fat-body was homogenized in 10 vol. of 0·88 M sucrose and the homogenate fractionated by differential centrifugation following (with minor modifications) the scheme of Hogeboom, Schneider & Pallade (1948). The flow sheet of a typical run is shown below. After the first centrifugation, an appreciable proportion of enzymic activity was retained in the lipid layer, but this was negligible after resuspension and recentrifugation, and the yield of particulate matter in fraction II was increased. The supernatant solution I obtained at the end was examined by phase-contrast microscopy and shown to contain very little particulate matter. The sediment in suspension III consisted of nuclei and cell debris, while the sediment in suspension II consisted mainly of small granular and rod-shaped bodies ranging in size from a fraction of a micron to 1 μ; these were seen to be isolated (i.e. no clumping had occurred). This suspension II can be regarded as containing

mitochondria-like bodies (Coupland, 1957). Suspension II may therefore provisionally be designated as the mitochondrial fraction. For investigation of enzymic activities, the supernatant solutions were dialysed against appropriate buffers, freeze-dried and redissolved in buffer; the sediments were resuspended in sucrose solution, centrifuged and resuspended in buffer solution.



Chromatographic technique. Amino-acids in deproteinized incubation mixtures were separated by applying aliquots of the mixture to Whatman no. 4 paper and developing either with *n*-propanol-water (80:20, v/v) or with phenol saturated with water. After drying, the amino-acid spots were detected by a ninhydrin spray. Incubation mixtures were deproteinized with two volumes of ethanol, centrifuged, the supernatant evaporated to dryness *in vacuo*, and the residue dissolved in water to the original volume. When the mixture contained cysteine, the incubation was done under nitrogen in a stoppered tube and deproteinized with ethanol containing a calculated excess of *N*-ethylmaleimide (Hanes, Hird & Isherwood, 1950). A semi-quantitative estimation of amino-acid concentration in incubation mixtures was obtained by running known amounts of the amino-acid in parallel and comparing visually the intensities of the spots. Chromatographic separation of keto-acids was carried out by the method of Cavallini, Frontali & Toschi (1949) as modified by Dagley, Fewster & Happold (1952).

Manometric techniques. Oxygen uptake was measured in the Warburg apparatus in the usual manner. Urea was estimated manometrically using urease, isolated from Jack bean meal, twice recrystallized following the method of Sumner &

Somers (1944). The urease activity of tissues was measured in the same manner in the presence of urea (300 μg./ml.).

Spectrophotometric technique. Measurements were made using a Unicam spectrophotometer (Model S.P. 500). The reagents were mixed in 10 mm. spectrophotometer cells, and the optical density measurements at the appropriate wavelength were taken at 1 min. intervals before and after addition of the enzyme or a substrate.

RESULTS

(1) *Transamination reactions*

It was shown that homogenates of fat-body were able to effect the synthesis of glutamate from added α-ketoglutarate by transfer of an amino-group from any one of a large number of other amino-acids. In a typical experiment, the homogenate was incubated with α-ketoglutarate and alanine, and samples withdrawn at zero time and at intervals during the next 300 min. and chromatograms of each run in parallel. The photograph of the chromatogram (Fig. 1) shows the progressive increase of glutamate with time and the simultaneous decrease in alanine. No glutamate was detected in control experiments in which one of three components (amino-acid, α-ketoglutarate or homogenate) was omitted. In a similar manner, it was shown that each of the following amino-acids could serve as the amino-group donor: glycine, L-aspartate, L-leucine, L-valine, DL-serine, DL-threonine, DL-phenylalanine, L-tyrosine, L-tryptophane, L-histidine, L-lysine, L-ornithine, L-arginine, L-cysteine, L-cystine and L-methionine. No synthesis of glutamate could be observed when the added amino-acid was L-proline or L-hydroxyproline.

The alanine/glutamate and aspartate/glutamate reactions were shown to be reversible. For example, Fig. 2 shows the progressive formation of alanine when fat-body homogenate was incubated with pyruvate and glutamic acid.

Semi-quantitative experiments showed that the transamination reactions had a broad pH optimum between 6·5 and 8·0 in phosphate buffer, and that there was detectable activity outside this range. When compared at pH 7·3, the glutamate/aspartate reaction was found to be the most rapid, synthesis of new amino-acid occurring initially at a rate of approximately 200 μmoles/g. fat-body (wet wt.)/hr. at 37° C. The alanine/glutamate reaction proceeded at about half this rate, and the reaction of α-ketoglutarate with the other amino-acids at speeds around one-twentieth of this rate.

The aspartate/glutamate and alanine/glutamate reactions were also catalysed by homogenates of Malpighian tubules and of mid-gut wall. Comparison on a wet-weight basis showed that the Malpighian tubules were more active than fat-body tissue and mid-gut wall rather less active. No significant differences were detected between the activities of adult and hopper fat-body tissue.

It was also shown that homogenates of these three tissues could effect the synthesis of alanine from pyruvate, utilizing glutamine as the amino-group donor, although the activity was less than when glutamate was used. Glutaminase I activity (using the nomenclature of Errara, 1949) which would hydrolyse glutamine

into glutamic acid, could not be detected; the alanine synthesis was probably due to a glutaminase II activity which has been shown by Meister & Tice (1950) to involve the transfer of the α-amino group of glutamine.

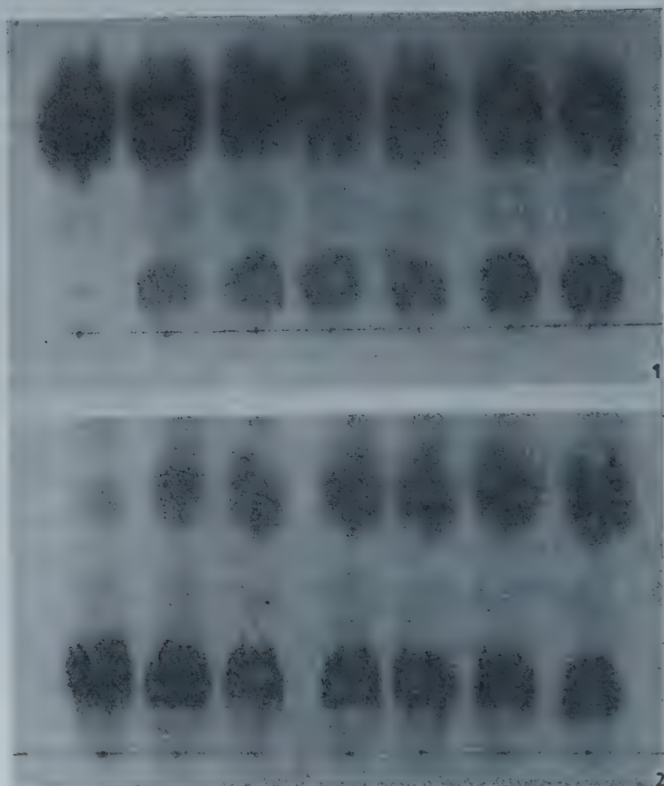


Fig. 1. Synthesis of glutamate from alanine and α-ketoglutarate by fat-body homogenate. Samples taken at 0, 30, 60, 90, 150, 210 and 300 min. from mixture incubated at 37° C. and initially containing L-alanine (0·05 M), α-ketoglutarate (0·05 M), pyridoxal phosphate (7×10^{-7} M) and fat-body (30 mg./ml.) in sodium potassium phosphate buffer (0·05 M, pH 7·3). Chromatograms run for 7 hr. in propanol/water and developed with ninhydrin. The upper spots are due to alanine, the lower to glutamate.

Fig. 2. Synthesis of alanine from glutamate and pyruvate by fat-body homogenate. Samples taken at 5, 15, 30, 45, 60, 120 and 180 min. Conditions as in Fig. 1, except that alanine and α-ketoglutarate in incubation mixture replaced by glutamate (0·05 M) and pyruvate (0·05 M).

Tests were made for a number of other possible transaminations. No transamination by fat-body homogenates could be detected when aspartate was incubated with phenylpyruvate, nor when oxalacetate was incubated with methionine, serine, lysine or leucine. A slow synthesis of alanine from pyruvate and aspartate was observed, but this did not occur when fat-body homogenate was dialysed before use and then fortified with pyridoxal phosphate; activity was restored by addition of catalytic amounts of glutamate to the system, indicating that the alanine synthesis

was probably due to the coupling in the undialysed system of two transaminases, aspartate/glutamate and glutamate/alanine by traces of endogenous glutamate. In contrast, alanine was formed from leucine and pyruvate in the presence of dialysed homogenates containing added pyridoxal phosphate, suggesting that a direct leucine/alanine transamination was operating. The rate of this reaction was similar to those of the slower glutamate transaminations.

Distribution of transaminase activity among cell fractions

Table 1 shows the ability of the four main fractions to synthesize glutamate from α-ketoglutarate and any one of ten amino-acids as amino-group donors. The activity of fraction III was low and may be attributed to whole cells which had escaped rupture and the washed lipide, layer IV, had negligible activity. As it is desirable

Table 1. *Synthesis of glutamate*

+ indicates that glutamate was detected chromatographically after incubation of the cell fraction with the amino-acid and α-ketoglutarate. ±, glutamate only just detectable.

Fraction	Components	Aspartate	Alanine	Leucine	Phenylalanine	Glycine	Tryptophane	Arginine	Ornithine	Serine	Lysine
I	Microsomes and soluble material	+	+	−	−	−	−	−	−	−	−
II	Mitochondria	+	+	+	+	+	+	+	+	+	+
III	Nuclei, etc.	+	+	±	−	−	−	−	−	−	−
IV	Lipide	±	±	−	−	−	−	−	−	−	−

Table 2. *Recovery of activities in cell fractions*

Figures give the amount of glutamate formed from α-ketoglutarate by four transaminations (μg. glutamate/g. original tissue/hr.)

Fraction	Transamination reaction			
	Aspartate/ glutamate	Alanine/ glutamate	Leucine/ glutamate	Phenylalanine glutamate
Whole homogenate	200	120	15	10
I. Supernatant	50	30	0	0
II. Mitochondria	100	70	10	5
III. Nuclei, etc.	30	10	Detectable	Detectable
IV. Lipide	Detectable	0	0	0

that reasonable recovery of the total initial activity should be obtained before making deductions about enzyme distribution, some measurements were made of the glutamate formed using the whole homogenate and the separate fractions (Table 2). Four different amino-acids were used as amino-group donors, and the glutamate was measured by the semi-quantitative chromatographic method. Each fraction was diluted with buffer to a volume equal to that of the original whole

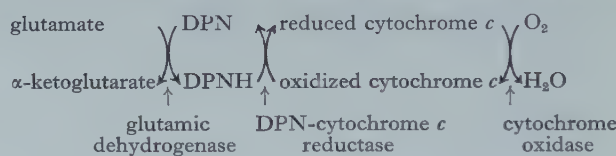
homogenate. The transaminase activities were measured in the presence of added pyridoxal phosphate, and they were much lower if this was omitted. The recoveries of activity are considered to be satisfactory.

(2) *Glutamine synthesis*

Fat-body, Malpighian tubule and mid-gut wall homogenates were shown to be capable of converting glutamic acid to glutamine. In a typical experiment, glutamate ($2 \cdot 5 \times 10^{-2}$ M), $MgSO_4$ (2×10^{-3} M), NH_4Cl (7×10^{-3} M), adenosine triphosphate (9×10^{-4} M) and fat-body homogenate (20 mg./ml.) were incubated in 5×10^{-2} M phosphate buffer, pH 7·3, at 37° C. Aliquots were withdrawn at intervals, deproteinized and chromatograms run in phenol/water. Under these conditions, glutamine was synthesized at a rate of about 100 μmoles/g. tissue (wet wt.)/hr. If any one of the four components added to the homogenate was omitted, no glutamine synthesis occurred.

(3) *Glutamic acid dehydrogenase system*

It is known that tissues of higher animals can effect the oxidation of glutamate to α-ketoglutarate, utilizing a pathway which may be expressed in a simplified form as



Since a similar system in fat-body would provide one way for the further metabolism of glutamate formed in transamination reactions from α-ketoglutarate, tests were made for enzymes which should be involved. Kilby & Hearfield (unpublished work) have shown that locust fat-body contains a cytochrome oxidase which is completely inhibited by cyanide.

DPN-cytochrome c reductase. Fat-body homogenates were shown to catalyse the reduction of cytochrome *c* by DPNH in the presence of potassium cyanide. The results of one experiment are shown in Fig. 3. When cyanide was omitted, no reduced cytochrome *c* accumulated as it was immediately reoxidized by the uninhibited cytochrome oxidase. No reduction occurred in the absence of DPNH. The slight fall in optical density was due to the separating out of lipide droplets in the homogenate. The rate of reduction of cytochrome *c* was calculated to be 7×10^{-9} mole/mg. tissue/5 min. at 10° C. (taking the extinction coefficient of reduced cytochrome *c* to be $2 \cdot 8 \times 10^{-7}$ cm.^{2}/mol.).

Glutamic acid dehydrogenase. Similar experiments in which the added DPNH was replaced by glutamate again led to the reduction of cytochrome *c*, at a rate corresponding to 5×10^{-10} mole/mg. tissue/5 min. at 10° C. Addition of either DPN or TPN did not increase the rate. When a mitochondrial preparation was used, a doubling of the rate of reduction was obtained on addition of DPN, but TPN was without effect. These findings suggest that the system as a whole requires DPN and

that there is some present in the mitochondrial preparation but insufficient for maximum enzymic activity. The endogenous DPN in the whole homogenate is, however, adequate. The identity of the product of the reaction, α-ketoglutarate, was checked by conversion to its 2:4-dinitrophenylhydrazone and running on a chromatogram in parallel with an authentic sample. Ninety per cent of the glutamic acid cytochrome *c* reductase activity of the whole homogenate was recovered in the mitochondrial fraction. Addition of Antimycin A to this fraction completely stopped the reduction of cytochrome *c* by added glutamate and DPN (Fig. 4).

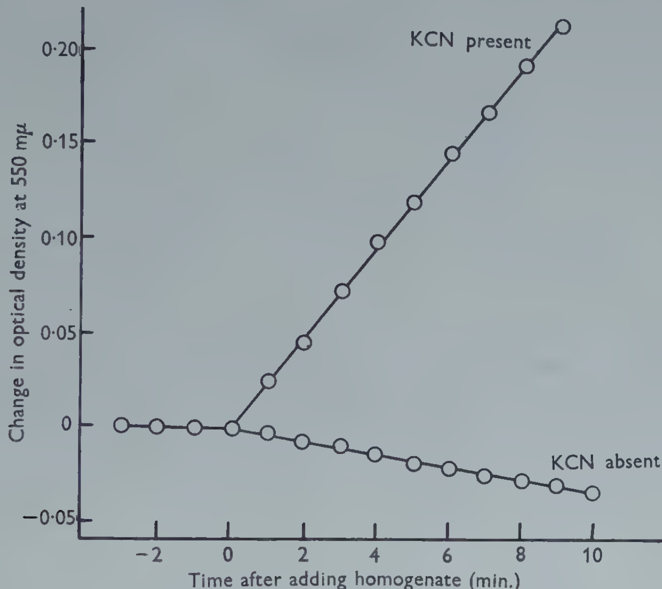


Fig. 3. DPNH-cytochrome *c* reductase of fat-body. Upper line shows increase of optical density due to the formation of reduced cytochrome *c* in mixture containing DPNH ($2\cdot5 \times 10^{-4}$ M), cytochrome *c* (0·3 mg./ml.), KCN (9×10^{-4} M) and fat-body homogenate (1·1 mg./ml.) in NaK phosphate buffer (0·1 M, pH 7·4). Lower line shows that no net reduction occurred when KCN omitted.

(4) *Arginase and the ornithine cycle*

During experiments on the transamination reactions catalysed by fat-body preparations, it was observed that incubation of homogenates with α-ketoglutarate and arginine gave rise to glutamate, together with another compound which reacted with ninhydrin. The same compound was also formed in control experiments in which ketoglutarate was omitted and in which no glutamate was formed. On chromatography, the compound was shown to move at the same rate as ornithine, both before and after elution and treatment with 7N-HCl for 24 hr. at 100° C. in a sealed tube. Manometric assay for urea on these incubation mixtures (after deproteinization with 66% ethanol) indicated the presence of urea, in amounts which would arise from the hydrolysis of arginine at a rate of 610 μmoles/g. fat-body (wet wt.)/hr. at 37° C. (Fig. 5). This high arginase activity was rather unexpected

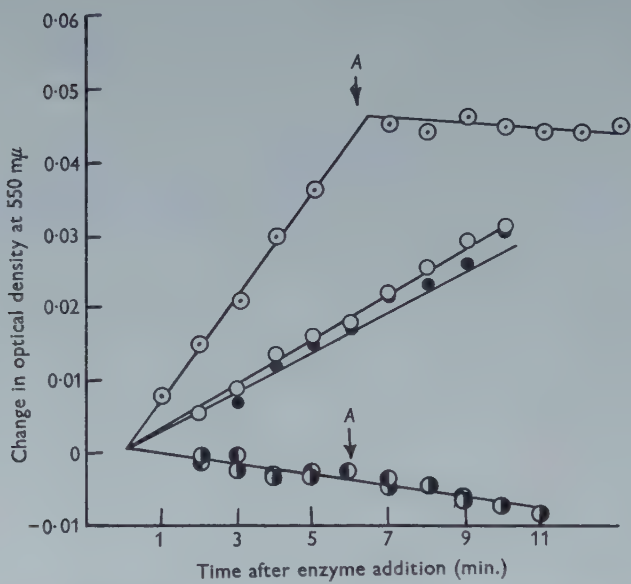


Fig. 4. Reduction of cytochrome *c* by mitochondrial fraction. Incubation mixtures contained mitochondria from 1·1 mg. of tissue per ml., nicotinamide (30 μg./ml.), semicarbazide (350 μg./ml.), KCN (7×10^{-4} M) in K phosphate buffer (0·1 M, pH 7·4) together with: ⊙, glutamate (6×10^{-3} M) + DPN ($1{\cdot}5 \times 10^{-4}$ M); ●, glutamate only; ◑, DPN ($1{\cdot}5 \times 10^{-4}$ M) only; ○, glutamate + TPN ($1{\cdot}5 \times 10^{-4}$ M); ◐, TPN only. Antimycin A (1 μg./ml.) added at *A*.

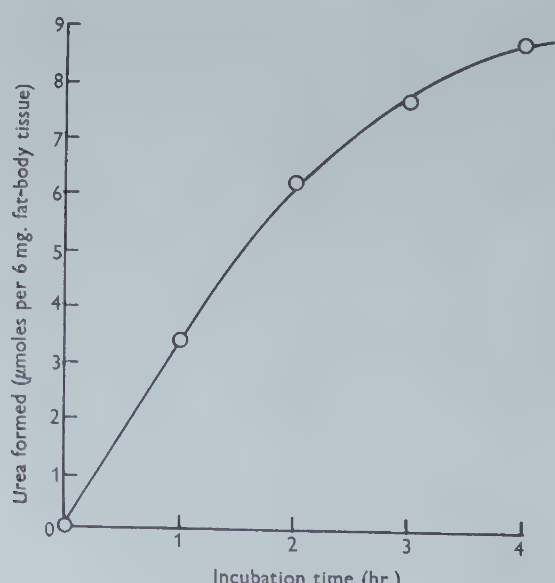


Fig. 5. Formation of urea when arginine is incubated with fat-body homogenates for various times.

since most insects excrete nitrogen largely in the form of uric acid and only very small amounts of urea have been detected in insect urines. Assay of fat-body and Malpighian tubule homogenates for urease failed to reveal any significant activity, the output of carbon dioxide from mixtures with and without added urea being almost negligible (about 1–2 μl./10 mg. tissue/hr. at 37° C.).

Table 3. *Amino-acid oxidase activity of fat-body homogenate*

Amino-acid added (0·03 M)	pH	Increase in O_2 uptake (μl./g. tissue/hr.)
DL-Alanine	9·0	1500
L-Alanine	9·0	160
DL-Valine	9·0	1500
L-Valine	9·0	150
DL-Leucine	9·0	950
L-Leucine	9·0	200
DL-Phenylalanine	9·0	1400
L-Phenylalanine	9·0	300
DL-Alanine	7·3	300
DL-Alanine	8·5	850
DL-Alanine	8·9	1530
DL-Alanine	9·0	1500
DL-Alanine	9·5	1150

(5) *Amino-acid oxidases*

The oxygen uptake of fat-body homogenates was slightly increased at pH 9 on the addition of L-alanine, L-valine, L-leucine or L-phenylalanine, but appreciably increased when the corresponding DL-amino-acids were added (Table 3). The optimum D-amino-acid oxidase activity with DL-alanine as substrate was around pH 9.

DISCUSSION

Intermediary metabolism in insects is a large and, for the most part, unexplored field. Not only is comparatively little known about the range of metabolic interconversions and degradations which are possible, or the biochemical pathways involved, but also the capabilities and relative importances of different tissues are largely undecided. Oenocytes, pericardial cells, mid-gut wall and Malpighian tubules have been shown to play some part in intermediary metabolism (see Wigglesworth, 1953), but it is the fat-body which has most frequently been proposed as being one of the major sites, and the suggestion has been made that it might be somewhat analogous to the mammalian liver. Much of the earlier work on metabolism in insects is rather fragmentary and often preparations of whole crushed insects have been examined for enzymic activity, so that no information was given about the location of the enzymes. Possible contamination of such preparations by food residues and micro-organisms from the gut was an undesirable feature. In the present work, we have largely confined ourselves to an investigation into some aspects of amino-acid metabolism, using preparations of fat-body from *Schistocerca*. This tissue was removed by dissection and was obtained as free from

other tissues as possible, but slight traces of the air-sac system were often unavoidably present; tests using tracheal tissue showed that this had negligible enzymic activity. It must be assumed that haemocytes were present since these are normally at rest on the surfaces of various organs. Auclair (1953) has suggested that the amino-acid oxidase activities which he observed with American roach fat-body tissue might be due to the intracellular symbionts which are always present. However, the enzymic properties described in this paper for *Schistocerca* fat-body cannot be due to this cause, since Coupland (1957) has shown that symbiotic bacteria are absent from locust fat-body cells.

The *in vitro* findings suggest that glutamate may play a central role in amino-acid metabolism in insects as in other organisms. As is usual, the two most active transaminations were:

$$\alpha\text{-ketoglutarate} + \text{alanine} \rightleftharpoons \text{glutamate} + \text{pyruvate},$$

$$\alpha\text{-ketoglutarate} + \text{aspartate} \rightleftharpoons \text{glutamate} + \text{oxalacetate},$$

but many other amino-acids were shown to react enzymically with α-ketoglutarate in a similar manner but at a slower rate. One route for the conversion of glutamate back to α-ketoglutarate would be through the mediation of the cytochrome-coupled glutamate dehydrogenase which was also found in the fat-body. Another pathway involving α-ketoglutarate metabolism would be the tricarbolic acid cycle, some of the enzymes of which have been shown to be present in insects (e.g. Watanabe & Williams, 1951; Spirites, 1951). Hearfield & Kilby (unpublished) have also shown that several of these enzymes are also present in the locust fat-body. The ability of homogenates to synthesize glutamine from glutamic acid is of interest in view of the high concentration of glutamine which has been found in the haemolymph of various insects (Levenbook, 1950) and which we have also found in locust blood. The main function of glutamine is probably as a reserve of amino-groups, but it can also function in some transamination reactions.

The activities of the transaminases involving glutamate were found to be of the same order of magnitude as those reported by Awapara & Seale (1952) for rat liver and kidney. The distribution of the transaminases in the cell fractions from fat-body is the same as that found by Hird & Rowsell (1950) for the transaminases of rat liver. The increase in activity of dialysed enzyme preparations on addition of pyridoxal phosphate is a good indication that the coenzyme of locust trans-aminases may well be the same as in mammalian and bacterial transaminases.

D-Amino-acid oxidases which have been found in the liver and kidney of many animals, in moulds and in bacteria, were also found in the locust, and, as in mammalian liver, were accompanied by a much weaker system for the aerobic oxidation of L-amino-acids. This may be a true L-amino-acid oxidase as in the mammal, or it might be the result of a coupling of a transaminase and a glutamate cytochrome *c* reductase; the observed reaction rates in fat-body would be sufficient to account for the observed oxygen uptake.

Cytochrome *c* was reduced ten times as fast by DPNH as by glutamate plus DPN, indicating that the glutamate-DPN stage was slower than the DPNH-

cytochrome *c* one. Since 90% of the activity of the coupled system (glutamate-DPN-cytochrome *c*) was recovered in the mitochondrial fraction, it would appear that the glutamic dehydrogenase is largely confined to the mitochondria. Hogeboom & Schneider (1953) have found that this enzyme was also concentrated in mammalian mitochondria. Sacktor (1953) has found a DPN-cytochrome *c* reductase using the housefly, and that this enzyme was confined to the mitochondria.

It is generally accepted that in mammalian systems there is an unidentified factor interposed in the electron transport pathway between DPNH and cytochrome *c* (Slater, 1950), and that this is inhibited by Antimycin A (Potter & Reif, 1952). The complete inhibition of cytochrome *c* reduction by fat-body mitochondrial fraction, glutamate and DPN when Antimycin A was added, indicates that the same or a similar factor is also involved in the insect system.

Under *in vitro* conditions, the high arginase and undetectable urease activity of fat-body preparations leads to an accumulation of urea when homogenates are incubated with arginine. Further work is in progress to try and decide whether or not urea is formed in the fat-body *in vivo*, and if so, whether it is hydrolysed by urease present elsewhere in the insect or is excreted unchanged. It is widely recognized that the demonstration of enzyme reactions *in vitro* is no sure indication that they are important in metabolic pathways in the whole animal. There is possibly a mechanism in the insect for the synthesis of arginine from citrulline, since it has been found (Hinton, Noyes & Ellis, 1951; Goldberg & de Meillon, 1948) that arginine in the diet of two insect species could be replaced to some extent by citrulline.

The over-all impression derived from the present preliminary survey is that the fat-body is not merely a passive organ for storage, but contains, *inter alia*, a range of enzymes active in amino-acid metabolism, and that the nature and distribution of these enzymes appears to resemble closely those in mammalian systems. Dr J. M. Stein, in a private communication, has drawn our attention to the work of Phipps (1950) who found that only a few shreds of fat-body remain in the adult *Locusta migratoria* by the time the eggs are fully developed, and she suggests that this might be evidence against the permanent contribution of the fat-body to intermediary metabolism. We have observed that the fat-body of *Schistocerca* behaves in a similar manner. An examination of the enzymic activities of these residual shreds would therefore be desirable.

SUMMARY

1. Homogenates of fat-body of *Schistocerca gregaria* Forsk. were shown to catalyse transamination reactions between α-ketoglutarate and numerous α-amino acids. The aspartate/glutamate and alanine/glutamate transaminases were the most active. They were present in both the 'soluble' and the mitochondrial fractions of fat-body cells and also in Malpighian tubules and mid-gut wall. The other transaminases in the fat-body were confined to the mitochondrial fraction.

2. Fat-body, Malpighian tubule and mid-gut wall homogenates were able to convert glutamic acid into glutamine, a compound which could also act as an amino-group donor in some transamination reactions.

3. A glutamate-cytochrome *c* reductase system which involved diphosphopyridine nucleotide was present in fat-body.

4. Fat-body contained an active arginase, but urease could not be detected. A D-amino-acid oxidase was present, together with a less active L-amino-acid oxidase.

5. In general, it appears that amino-acid metabolism in the locust resembles that in higher animals.

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REFERENCES

AGRELL, I. (1949). Occurrence and metabolism of free amino acids during insect metamorphosis. *Acta physiol. scand.* **18**, 247–58.

ALBRITTON, E. C. (1955). *Standard Values in Nutrition and Metabolism.* Philadelphia: W. P. Saunders Co.

AUCLAIR, J. L. (1949). Thesis, Cornell University Library, Ithaca, New York.

AUCLAIR, J. L. (1953). Amino-acids in insects. *Canad. Ent.* **85**, 63–8.

AWAPARA, J. & SEALE, B. (1952). Distribution of transaminases in rat organs. *J. Biol. Chem.* **194**, 497–502.

BARRON, E. S. G. & TAHMISIAN, T. N. (1948). The metabolism of cockroach muscle. *J. Cell. Comp. Physiol.* **32**, 57–76.

BEISENHERZ, G., BOLTZE, H. J., BÜCHER, T. H., CZOK, R., GARBADE, H. K., MEYER-ARENDT, E. & PFLEIDERER, G. (1953). Diphosphofructose-Aldolase, Phosphoglyceraldehyd-Dehydrogenase, Milchsäure-Dehydrogenase, Glycerophosphat-Dehydrogenase und Pyruvat-Kinase aus Kaninchenmuskulatur in einem Arbeitsgang. *Z. Naturf.* **8*b***, 555–77.

BODENSTEIN, D. (1946). Investigation on the locus of action of DDT in flies (*Drosophila*). *Biol. Bull., Woods Hole*, **90**, 148–57.

CAVALLINI, D., FRONTALI, N. & TOSCHI, G. (1949). Determination of keto acids by partition chromatography on filter paper. *Nature, Lond.*, **163**, 568–9.

COUPLAND, R. E. (1957). Observations on the normal histology and histochemistry of the fat body of the locust (*Schistocerca gregaria*). *J. Exp. Biol.* **34**, 290–6.

DAGLEY, S., FEWSTER, M. E. & HAPPOLD, F. C. (1952). The bacterial oxidation of phenylacetic acid *J. Bact.* **63**, 327–36.

ERRARA, M. (1949). Liver glutaminases. *J. Biol. Chem.* **178**, 483–93.

GOLDBERG, L. & DE MEILLON, D. (1948). Nutrition of the larva of *Aëdes ægypti* Linnæus. Protein and amino-acid requirements. *Biochem. J.* **43**, 379–87.

HANES, C. S., HIRD, F. J. R. & ISHERWOOD, F. A. (1950). Synthesis of peptides in enzymic reactions involving glutathione. *Nature, Lond.*, **166**, 288–92.

HINTON, T., NOYES, D. T. & ELLIS, J. (1951). Amino acids and growth factors in a chemically defined medium for *Drosophila*. *Physiol. Zoöl.* **24**, 335–53.

HIRD, F. J. R. & ROWSELL, E. V. (1950). Additional transaminations by insoluble particle preparations of rat liver. *Nature, Lond.*, **166**, 517–8.

HOGEBOOM, G. H. & SCHNEIDER, W. C. (1953). Intracellular distribution of enzymes. XI. Glutamic dehydrogenase. *J. Biol. Chem.* **204**, 233–8.

HOGEBOOM, G. H., SCHNEIDER, W. C. & PALLADE, G. E. (1948). Isolation of intact mitochondria from rat liver. *J. Biol. Chem.* **172**, 619–37.

HUNTER-JONES, P. (1956). *Instructions for Rearing and Breeding Locusts in the Laboratory*. London: Anti-Locust Research Centre.

KILBY, B. A. & NEVILLE, E. (1956). Amino acid metabolism in locust tissues. *Biochim. Biophys. Acta*, **19**, 389–90.

LEVENBOOK, L. (1950). The composition of horse bot fly larva blood. *Biochem. J.* **47**, 336–46.

LIPKE, H. & FRAENKEL, G. (1956). Insect nutrition. *Ann. Rev. Entomol.* **1**, 17–44.
MEISTER, A. & TICE, S. V. (1950). Transamination from glutamine to α-keto acids. *J. Biol. Chem.* **187**, 173–87.
PATTON, R. L. (1953). Excretion. *Insect Physiology*, pp. 387–403. Ed. K. D. Roeder. New York: Wiley.
PHIPPS, J. (1950). The maturation of the ovaries and the relation between weight and maturity in *Locusta migratoria* migratorioides (R & F). *Bull. ent. Res.* **40**, 539–57.
POTTER, V. R. & REIF, J. (1952). Inhibition of an electron transport component by Antimycin A. *J. Biol. Chem.* **194**, 287–97.
SACKTOR, B. (1953). Investigations on the mitochondria of the housefly, *Musca domestica* L. II. Oxidative enzymes with special reference to malic oxidase. *Arch. Biochem. Biophys.* **45**, 349–65.
SLATER, E. C. (1950). The components of the dihydrocozymase oxidase system *Biochem. J.* **46**, 484–99.
SPIRITES, M. A. (1951). Demonstration of the presence of Krebs cycle enzymes in *Drosophila melanogaster*. *Fed. Proc.* **10**, 251.
SUMNER, J. B. & SOMERS, G. F. (1944). *Laboratory Experiments in Biological Chemistry*. New York: Academic Press.
WATANABE, M. I. & WILLIAMS, C. M. (1951). Mitochondria in the flight muscles of Insects. I. Chemical composition and enzyme content. *J. gen. Physiol.* **34**, 675–89.
WIGGLESWORTH, V. B. (1953). *The Principles of Insect Physiology*, 5th ed. with addenda. London: Methuen.

OBSERVATIONS ON THE NORMAL HISTOLOGY AND HISTOCHEMISTRY OF THE FAT BODY OF THE LOCUST (*SCHISTOCERCA GREGARIA*)

By R. E. COUPLAND
Department of Anatomy, University of Leeds

(*Received* 7 *March* 1957)

(With Plate 5)

INTRODUCTION

The existence of intracellular inclusions of bacteria, rickettsia and virus particles in insects has been recognized for many years, and the subject has been reviewed by Steinhaus (1946). Micro-organisms have been described in the wall of the gut, the Malpighian tubules, the genital organs, the blood and the fat body of a variety of insects. They may exist in these sites as a result of a disease process which may result in the death of the host or as normal symbionts, as in the case of the inclusions in the fat body of the cockroach (Gier, 1936). D'Herelle (1911), investigating the high mortality rate of the locust *Schistocerca pallens*, reported the presence of the micro-organism *Coccobacillus acridiorum* in the intestinal canal of the dead locust. Pospelov (1926) observed the same micro-organism in the blood of the locust *L. migratoria*, but considered that it was a normal inhabitant which only became pathogenic when the insects were reared in unfavourable conditions of temperature and humidity. The recent work of Kilby & Neville (1957) has indicated that the fat body of the locust possesses marked enzymic activity, and the present work has been undertaken in an attempt to find out whether this activity is the result of the presence of symbiotic micro-organisms or to some normal cell constituent.

MATERIALS AND METHODS

Healthy specimens of the desert locust *Schistocerca gregaria* (1st- to 5th-instar stages and adults) have been examined as fresh preparations and after fixation. Material has been fixed in 10% formalin, Carnoy (6:3:1), Flemming, Champy, absolute alcohol, formol-dichromate, formol-calcium-cobalt (McManus, 1946), alcoholic Bouin and aqueous Bouin. Some paraffin embedded sections (cut at 4–8 μ) have been stained by a variety of histological methods, including haematoxylin and eosin, iron haematoxylin, Giemsa, Gram's stain, Bensley's acid-aniline methyl green (Cowdrey, 1948), Meyer's haemalum and ammoniacal silver nitrate; other sections have been stained for protein by the Millon and Danielli methods (Lillie, 1954), for glycogen by the P.A.S. technique (Hotchkiss, 1948), with saliva and acetylation controls (Gersch, 1949), for DNA and RNA by the Feulgen and methyl

green pyronin methods (Pearse, 1953) using perchloric acid as a control, and for phospholipids by the Sudan Black B method of McManus (1946).

Fresh tissue was examined by phase contrast and by polarized light, and also after being stained supravitally by Janus Green B and neutral red.

FINDINGS

Observations on fixed tissues

(*a*) *General morphology and histology*

The fat body extends throughout the abdominal and thoracic cavities. It can be divided into a peripheral portion which is firmly attached to the overlying epidermis, and a more central mass which exists as a loose meshwork of anastomosing lobes in the space between the gut and body wall (Pl. 5, fig. 1). The peripheral and central masses of fatty tissue are continuous at many points and are intertwined with genital and Malpighian tubules. The fat body exists as a single or double layer of cells in the 1st-instar stage; at this time a few small discrete fat droplets may be seen scattered in the cytoplasm which, after staining with haematoxylin and eosin or Giemsa, is both eosinophilic and basophilic—the eosinophilia being diffuse, the basophilic staining reaction being confined to a fine reticular stroma. Fat cells increase in number by mitotic division and by the 2nd-instar stage are engorged with fat droplets so that the non-fatty cytoplasm is mainly confined to the perinuclear region, from which protoplasmic strands radiate towards the cell membrane. The fat cells contain intensely chromatic nuclei 10–12 μ in diameter; the cell membrane is often difficult to define. The fat cells are intimately associated with oenocytes, which are most numerous in the peripheral parts of the fat body but are also occasionally observed in the more central region. The oenocytes are large cells of 60–75 μ in diameter, each having a single rounded nucleus 15–20 μ in diameter whose chromatin is in smaller clumps and is less dense than that of the fat cells; the cytoplasm is diffusely eosinophilic but is permeated by a fine basophilic network. A variable number of small vacuoles may be observed in the cytoplasm of the oenocytes (Pl. 5, figs. 4, 5). The association between fat cells and oenocytes persists throughout all developmental stages and the two types of cell are present in the mature adult. Oenocytes are always most numerous in the subepidermal region and they retain their typical form throughout the life cycle of the locust; in the old adult some yellow granular pigment and birefringent uric acid deposits, which are stained by haemalum, are often observed in the cytoplasm of these cells.

Tissues fixed in formol dichromate and stained with Bensley's acid fuchsin-methyl green (Cowdrey, 1948) show the presence of small (0·1–0·5 μ) red granules, which may be rounded or ovoid, in the cytoplasm of the fat cells (Pl. 5, fig. 2), the Malpighian tubules, the gut and the follicular cells of the ovaries; these structures have not been observed in the oenocytes, but this may be due to the heavy background staining of the cytoplasm which masks the reaction. In addition to these small structures larger cellular inclusions in the Malpighian tubules and gut are also stained by acid fuchsin; these larger elements are probably secretion granules

as similar structures may be observed in the lumen of the Malpighian tubules. The 'albuminoid' plaques as described by Hollande (1914) in the fat body of *Vanessa* have not been observed in the locust.

Tissue fixed in absolute alcohol or Carnoy was stained for uric acid by the ammoniacal silver nitrate method (Hollande, 1914), and for urates by Mallory's haemalum method (Lillie, 1954). Scattered deposits of silver are observed in both oenocytes and fat cells at all stages, the number of granules increasing with age. No haemalum-positive granules were observed during the instar stage, but a few are present in the oenocytes of the adult locust.

No micro-organisms have been observed in sections stained by either Giemsa or Gram's method for tissue sections.

(*b*) *Histochemical methods*

(1) *Nucleoproteins.* Using Carnoy-fixed material stained by the Feulgen method DNA is confined to the nuclei in all cells.

In tissue fixed with 10% formol RNA-positive material is present in large amounts and scattered throughout the cytoplasm of the oenocytes and 1st-instar fat cells; in older specimens it is abundant throughout the oenocytes but is mainly confined to the perinuclear region of the fat cells. RNA is reduced in amount in the adult insect.

(2) *Glycogen.* The non-fatty cytoplasm of 1st- to 5th-instar fat body cells contains large amounts of glycogen; this is present in both fat cells and oenocytes (Pl. 5, fig. 3). The reaction is in part diffuse and in part granular. The glycogen content of the adult locust is less than that of the immature insect and usually exists as small granules.

(3) *Proteins.* Both the Danielli diazo reaction and the Millon reaction are positive throughout the non-fatty cytoplasm of the fat body cells and oenocytes (Pl. 5, fig. 4). The reaction is partly diffuse and is partly concentrated in small cytoplasmic granules; large plaques of protein are not observed. The reaction is less intense in the adult locust.

(4) *Fat.* In Champy-fixed material reduced osmic acid occupies practically the whole of the cytoplasm of the fat-engorged cells. In sections fixed with formol-calcium-cobalt and stained with Sudan Black show an intense staining of the cytoplasm of the oenocytes with only a slight reaction in the fat cells (Pl. 5, fig. 5).

Stains for bacteria

Blood smears and smears of crushed whole fat body and of homogenized centrifuged material have been examined, after heat fixation, for bacteria. Smears were stained by Gram's method and by Giemsa. No bacteria or other micro-organisms were observed in any specimen.*

* The author is indebted to Dr K. Zinneman of the Department of Bacteriology, University of Leeds, for performing Gram stains and examining the sections.

Fresh tissue

Under the ordinary light microscope, and with phase contrast, the majority of the cells of the fat body are seen to be engorged with fat droplets of various sizes from less than 1–10 μ in diameter. In the immature forms the fat is pigmented and has a yellow colour; this colour disappears in a patchy fashion in the young adult and the old adult is practically devoid of pigment. In addition to fat droplets, small rounded granules of about 0·5 μ in diameter may be observed scattered throughout the cytoplasm of the cells; many of these show Maltese cross-birefringence and are considered to be uric acid spherules. Similar granules, together with birefringent rectangular crystals of up to 1 μ in long axis, are also seen in the cells of the Malpighian tubules. The rectangular crystals are never observed in the fat body. The fat droplets are monorefringent. The application of absolute alcohol to the tissue results in the disappearance of the Maltese cross-birefringent granules and the appearance of sheaves of uric acid crystals which can be seen by polarized light or by staining with Meyer's haemalum.

Supravital staining

Tissues were stained supravitally with 1:10,000 Janus Green B in 0·9% saline. Shortly after the application of the dye the nuclei of the haemocytes and the general cytoplasm of the cells of the Malpighian tubules become blue. The fat body has no affinity for the dye during the first 10–15 min. After this time the nuclei of the haemocytes may be seen stained as before, but diffuse coloration of the cells of the Malpighian tubules is no longer apparent and the dye is now seen to be concentrated in small round or ovoid perinuclear bodies which measure 0·1–0·5 μ in long axis, the remainder of the cytoplasm being either uncoloured or pale pink as a result of the formation of leuco Janus Green B or diethyl safranin (Lazarow & Cooperstein, 1953). In the cells of the fat body (fat cells and oenocytes) and the follicular cells of the ovary small discrete blue stained bodies 0·2–0·6 μ in diameter and round or ovoid in shape may now be observed. These are often most numerous in the perinuclear region, but are also scattered throughout the non-fatty parts of the cytoplasm. These granules are non-refractile and hence can readily be distinguished from small refractile fat droplets which at times appear to have a slight affinity for the dye; they are monorefringent. The Janus Green-positive granules are not stained by either Meyer's haemalum or an alcoholic solution of iodine. They are therefore distinct from uric acid granules, glycogen and small fat droplets. After about 40 min. the blue colour fades.

The application of a mixture of Janus Green 1:10,000 and neutral red 0·5% in saline to fresh tissue results after an interval of 10–15 min. in the staining of the small rounded or ovoid bodies, described above, with Janus Green, whilst larger cellular inclusions 0·5–1 μ show an affinity for neutral red. The nuclei of the fat cells and other tissues are stained intensely with neutral red.

DISCUSSION

The application of routine bacteriological stains to both smears and tissue sections has failed to reveal the presence of bacteria in the fat body of the locust. Virus particles of the type described by Williams & Smith (1957) in the crane fly *Tipula paludosa* can be excluded, as no DNA-positive material has been observed in the cytoplasm of the cells. It would therefore seem that the fat cells themselves, or the associated oenocytes, or both, must be responsible for the enzymic activity observed by Kilby & Neville (1957) in the carefully dissected fat body of the 5th-instar locust.

It has been shown by Lazarow & Cooperstein (1953) that in mammals supravital staining by Janus Green B is an oxygen-dependent reaction which depends upon the enzyme activity of the cell; the dye is thought to be reduced enzymically in the non-mitochondrial portion of the cell and more slowly in the mitochondria because of the presence of cytochrome oxidase in these structures. The application of a 1:10,000 solution of Janus Green B to portions of intact fat body, Malpighian tubules and follicular cells of the ovaries is followed after an interval of 10–15 min. by the staining of small rounded or ovoid structures (0·1–0·5 μ in diameter) in the cytoplasm of these cells. The structures are often most numerous in the perinuclear region and the colour fades after 30–45 min. These small granules are neither uric acid, minute fat droplets, nor glycogen, as judged by their birefringence and staining with Meyer's haemalum, by their non-refractile nature or by staining with iodine. Similar small acid aniline-positive structures may be stained along with larger secretion droplets and cellular inclusions by Bensley's acid aniline-methyl green; this stain is often considered to be relatively specific for mitochondria in tissue sections (Cowdrey, 1948). The fact that the cellular inclusions are stained by both Janus Green B and acid fuchsin (whilst not being identical in either microscopic appearance or staining with uric acids or urates, glycogen or fat) would suggest that these elements may be a form of mitochondrion; if so, they are more rounded or ovoid than the typical mammalian forms, as observed in the liver or pancreas, and more closely resemble the round or ovoid bodies described by Farrant, Potter, Robertson & Wilkins (1956) in the red beet.

Although accurate counts of the mitochondria-like bodies in the fat body cells cannot be made, because of the presence of overlying fat droplets, there does not appear to be any definite change in number in the successive developmental stages. This constancy in number would be more in keeping with the bodies being true organoids rather than inclusions, for both the uric acid and glycogen content of the cells vary in the different developmental stages.

The fat body of the locust is very rich in glycogen during the 1st- to 5th-instar stages. This is present not only in fat cells but also in oenocytes; the presence of glycogen in the latter type of cell is in contrast with the conditions found in the fat body of the mosquito larvae (Wigglesworth, 1942). Protein and phospholipids are very abundant in the oenocytes, while only relatively small quantities are present in the fat cells.

In conclusion, it would seem, therefore, that the enzymic activity of the fat body of the locust is due to the presence of mitochondria-like bodies in both fat cells and associated oenocytes. As the great majority of the cells of the more readily dissected portion of the fat body are true fat cells, the oenocytes being most numerous at the periphery, it is probable that the activity observed by Kilby & Neville (1957) was mainly due to the presence of these structures in the true fat cells.

SUMMARY

The fat body of the locust *Schistocerca gregaria* is composed of both fat cells and oenocytes. The cells increase in number by mitotic division. Glycogen, proteins and phospholipids are present in all cells but are most abundant in the oenocytes; the amounts of these substances are less in the adult than in the immature forms, whereas uric acid is more abundant in the adult.

Small mitochondria-like structures (as judged by their staining reactions to Janus Green B and acid-fuchsin-methyl green) have been observed in the cytoplasm of the fat cells, the oenocytes, the Malpighian tubules and the follicular cells of the ovary.

No micro-organisms have been observed.

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REFERENCES

Cowdrey, E. V. (1948). *Laboratory Technique in Biology and Medicine*. Baltimore: Williams & Wilkins Co.

Farrant, J. L., Potter, C., Robertson, R. N. & Wilkins, M. J. (1956). The morphology of red beet (*Beta vulgaris* L.) mitochondria. *Aust. J. Bot.* **4**, 117–24.

Gersh, I. (1949). A protein component of the Golgi apparatus. *Arch. Path.* (*Lab. Med.*), **47**, 99–109.

Gier, H. T. (1936). The morphology and behaviour of the intracellular bacterioids of roaches. *Biol. Bull., Woods Hole*, **71**, 433–52.

d'Herelle, F. (1911). Sur une épizootie de nature bactérienne sévissant sur les sauterelles au Mexique. *C. R. Acad. Sci., Paris*, **152**, 1413–15.

Hollande, A-Ch. (1914). Formations endogènes des cristalloïdes albuminoïdes et des urates des celles adipeuses des chenilles de *Vanessa* io et *Vanessa urticae*. *Arch. Zool. exp. gén.* **53**, 559–78.

Hotchkiss, R. D. (1948). A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. *Arch. Biochem.* **16**, 131–41.

Kilby, B. A. & Neville, Elizabeth (1957). Amino-acid metabolism in locust tissues. *J. Exp. Biol.* (in the Press).

Lazarow, A. & Cooperstein, S. J. (1953). Studies on the enzymatic basis for the Janus Green B staining reactions. *J. Histochem. Cytochem.* **1**, 232–41.

Lillie, R. D. (1954). *Histopathologic Technic and Practical Histochemistry*. New York: Blakiston Co. Inc.

McManus, J. F. A. (1946). Demonstration of certain fatty substances in paraffin sections. *J. Path. Bact.* **58**, 93–5.

Pearse, A. G. E. (1953). *Histochemistry, Theoretical and Applied*. London: J. and A. Churchill Ltd.

Pospelov, V. P. (1926). The influence of temperature on the maturation and general health of *Locusta migratoria* L. *Bull. Ent. Res.* **16**, 363–67.

Steinhaus, E. A. (1946). *Insect Microbiology*. Ithaca, N.Y.: Comstock Publishing Co. Inc.

Wigglesworth, V. B. (1942). The storage of protein, fat, glycogen and uric acid in the fat body and other tissues of mosquito larvae. *J. Exp. Biol.* **19**, 56–77.

Williams, R. C. & Smith, K. M. (1957). A crystallizable insect virus. *Nature, Lond.*, **179**, 119–20.

EXPLANATION OF PLATE 5

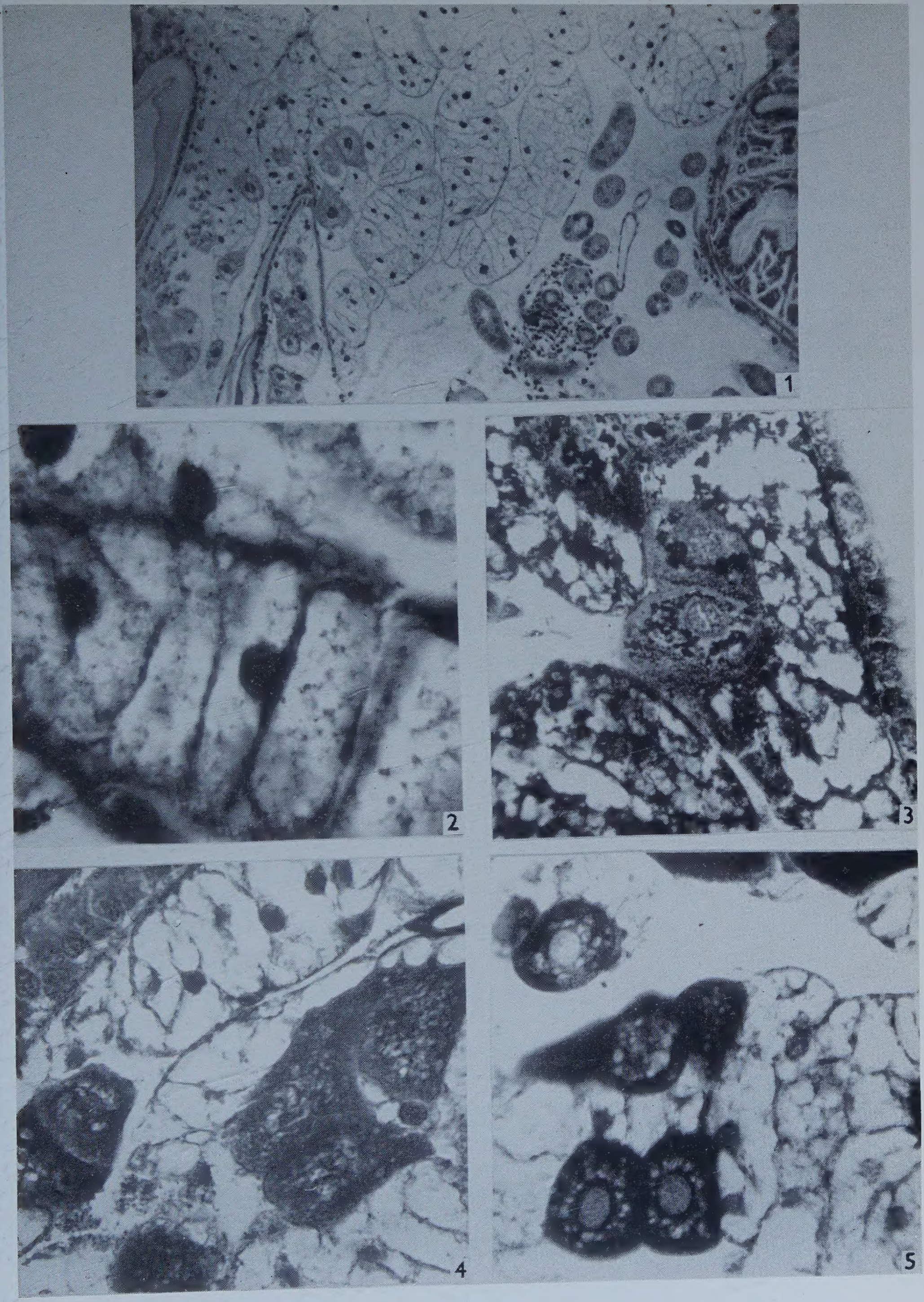
Fig. 1. Fat body of 4th-instar locust. The body wall lies at the left and the gut at the right extremity of the photomicrograph. A few oenocytes lie in the peripheral zone. Haematoxylin and eosin, × 90.

Fig. 2. Fat cells of 4th-instar locust. Small discrete acid-fuchsin stained bodies are scattered throughout the cell. Acid-fuchsin-methyl green, × 1250.

Fig. 3. 4th-instar locust. Fat cells and oenocytes stained for glycogen. PAS, × 400.

Fig. 4. 4th-instar locust. Fat cells and oenocytes stained for protein. Danielli method, × 400.

Fig. 5. 4th-instar locust. Fat cells and oenocytes stained by McManus's method for phospholipid. Sudan Black B, × 400.



COUPLAND—OBSERVATIONS ON THE NORMAL HISTOLOGY AND HISTOCHEMISTRY OF THE FAT BODY OF THE LOCUST (*SCHISTOCERCA GREGARIA*)

(*Facing p.* 296)